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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Arif, M. I. (2018). *Engineering amidases for peptide C-terminal modification*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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Chapter 1

General Introduction: Enzymatic Synthesis of Bioactive Peptides

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Parts of this chapter have been published in:

Toplak, A., Arif, M. I., Wu, B. & Janssen, D. B. Discovery and Engineering of Enzymes for Peptide Synthesis and Activation. Green Biocatalysis (John Wiley & Sons, Inc., 2016).

Part I. Peptides: properties, activity, and applications

1.1 Peptides - General aspects

Peptides and proteins form a highly diverse group of macromolecular compounds consisting of chains of α-amino acids linked by amide bonds between the carboxylate groups and amino groups. Proteins and peptides have the same general chemical structure, but the name 'peptides' is often restricted to chains with an upper size limit of about 50 amino acids, or a molecular mass of about 6,000 Da [1]. While large proteins often act as catalysts (enzymes) or structural proteins (keratin, collagen), smaller naturally occurring peptides are more usually involved in regulatory processes in organisms. The diversity of such peptides is enormous, their occurrence is widespread, and they play essential roles in processes such as regulation of metabolic activity, immune response, hormone secretion and cellular defense. Probably the most known example is the hormone insulin, which is composed of 2 cross-linked chains of 21 and 30 amino acids with a total mass of 5773 Da, and controls serum glucose levels. Other peptide-mediated activities include cell to cell communication, control of neurological processes (neurotransmitters, neuromodulators, e.g. influencing pain sensation), and regulation of fertility [2,3].

The multitude of activities attributed to peptides has sparked widespread interest in developing and using peptides in different industries and biomedical applications. This varies from a simple dipeptide aspartame (Asp-Phe-OCH_3) which is used as an artificial sweetener in foods and drinks, to numerous large peptides that influence metabolism, cell proliferation, or other processes. The use of peptides as therapeutic drugs is attractive because of their high activity and specificity, as well as low toxicity (with exceptions) and low tendency to accumulate in tissues. Synthetic peptides can also be used as antigens, enzyme substrates, and inhibitors that influence signaling pathways in biomedical research. They may be used as probes for *in vivo* diagnostic purposes. Peptides or peptide tags can facilitate protein purification when used as immobilized affinity ligands and can interfere with protein-protein interactions. Peptides are also developed for use in molecular electronic devices [1,2].

The importance of peptides in healthcare, nutrition, and cosmetics triggered widespread interest in methodologies for peptide synthesis and characterization. However, production of peptides at large scale is challenging at this moment, and efficient platforms for cost-efficient and environment-friendly production of peptides are still in high demand [4,5]. The use of enzymes for peptide synthesis is of special interest since it avoids harsh reaction conditions that may lead to the formation of unwanted side products. Furthermore, peptides often need to be modified to improve their application potential [6]. This is especially true for pharmaceutical peptides where modification influences pharmacokinetics and interaction with target molecules. The use of enzymes

for peptide synthesis and especially modification is the topic of this thesis and is further examined in this introductory chapter, after discussing the use of peptides in various applications in more detail.

1.2 Therapeutic peptides

In the pharmaceutical industry, peptide-based therapeutic agents comprise the fastest growing market segment. Currently, 68 approved peptide-based drugs are commercialized, several of which generate multibillion-dollar sales annually [7]. The majority of these peptides consist of about 8-10 amino acids. The market for therapeutic peptides is projected to surpass US$ 70 billion in 2018-2019, corresponding to an annual growth rate of 9 to 10%, which is faster than that of other pharmaceuticals[8]. Furthermore, peptide-based drugs currently represent over 15% of the total number of new entities registered by the U.S. Food & Drug Administration (FDA) [9,10]. Over 300 companies are involved in developing 292 peptide-based drugs in 780 oncology projects, according to a survey of ClinicalTrials.gov [11,12]. In 2018, around 500 to 600 peptides are in preclinical development, while 155 peptides are examined in clinical trials and 97% of them are expected to pass the regulatory phase [7,13]. Most of these trials are targeting cancer, metabolic diseases, and cardiovascular problems [7,8]. The success rate is nearly twice as that of the small molecule drugs [13,14].

Most peptide therapeutics target metabolic disorders, fertility problems, cancer, and problems associated with the central nervous system. Examples of peptide therapeutics that have entered the market include glatiramer acetate (Copaxone, 10 amino acids) used for treatment of multiple sclerosis, the gonadotropin antagonist leuprorelin (Lupron, 9 amino acids), exenatide (Byetta, 39 amino acids) for type II diabetes, and enfuvirtide (T-20/Fuzeon, 36 amino acids), which inhibits HIV-1 membrane fusion [8]. More examples of recently introduced pharmaceutical peptides are listed in Table 1. Peptide therapeutics are also being developed for cardiovascular disorders, infection management (see next section), hematological disorders, gastrointestinal disorders, dermatological problems, respiratory disorders, and hormone metabolism.[12] Following the sequencing of the human genome, peptides have become an important focus of biotechnological research due to the increasing awareness of their key role in regulation and immunity. The discovery of new targets with which peptides can interfere and help to treat diseases and modulate the immune system is steadily growing [15,16].

Table 1. Peptide therapeutics approved from 2003 to 2017 [7,17–20]

Peptide	Peptide length	Indication	Activity	Approval Date
Enfuvirtide	36	AIDS	Protein-protein inhibition	2003
Daptomycin	11	Bacterial skin infections	Plasma membrane synthesis inhibition	2003
Abarelix	10	Prostate cancer	LH-RH[a] agonist	2003
Human secretin	27	Diagnostic for pancreatic function	Pancreatic/Gastric secretion stimulation	2004
Ziconotide	25	Chronic pain	N-type Ca Channel blocker	2004
Pramlintide	37	Types 1 and 2 diabetes	Calcitonin agonist	2005
Exenatide	39	Type 2 diabetes	GLP-1[b] receptor agonist	2005
Lanreotide	8	Acromegaly	SST agonist	2007
Icatibant	10	Hereditary angioedema	Bradykinin B2 receptor antagonist	2008
Degarelix	10	Prostate cancer	GnRH[c] antagonist	2008
Mifamurtide	2	Osteosarcoma	Immunostimulant	2009
Ecallantide	60	Hereditary angioedema	Plasma kallikrein inhibitor	2009
Liraglutide	30	Type 2 diabetes	GLP-1 receptor agonist	2010
Tesamorelin	44	HIV lipodystrophy	GHRH[d] analog	2010
Sinapultide	21	Respiratory distress syndrome	Surfactant	2012
Peginesatide	40	Anemia	Erythropoietin analog	2012
Carfilzomib	4	Multiple myeloma	Proteasome inhibitor	2012
Linaclotide	14	Irritable bowel syndrome	Guanidyl cyclase 2C agonist	2012
Pasireotide	6	Cushing's disease	Somatostatin analog	2012
Teduglutide	33	Short bowel syndrome	GLP-2 analog	2012
Lixisenatide[e]	44	Type 2 diabetes	GLP-1 receptor agonist	2013
Albiglutide	60	Type 2 diabetes	GLP-1 receptor agonist	2014
Dulaglutide	30	Type 2 diabetes	GLP-1 receptor agonist	2014
Etelcalcetide	8	Secondary hyperparathyroidism	Calcimimetic agent	2016
Plecanatide	16	Chronic idiopathic constipation	Guanylate cyclase C agonist	2017
Abaloparatide	34	Osteoporosis	Parathyroid hormone related peptide (PTHrP) analog	2017

[a] Luteinizing hormone-releasing hormone
[b] Glucagon-like peptide-1
[c] Gonadotropin-releasing hormone
[d] Growth hormone-releasing hormone
[e] Lixisenatide was approved in the US in 2016 and in Europe in 2013

1.2.1 Anti-infective peptides

Over the past seven decades, antibiotics have saved numerous lives and contributed to the growing life expectancy of humans. Nevertheless, due to emerging antibiotic resistance and increased regulations concerning side effects, new anti-infective therapies have to be developed. The problem is serious; it has been predicted that more deaths will be caused by drug-resistant bacterial infections than by cancer in 2050 [21]. Widespread resistance could result in bringing humans back to the pre-antibiotic world [22]. Consequently, there is a growing demand for non-conventional approaches to treating infections caused by pathogenic bacteria. Peptide-based antibiotics are deemed to fill the void created by growing antibiotic resistance. Therefore, studies towards pharmacological development of peptides are on the rise [23]. The use of antimicrobial peptides (AMPs), either synthetic or of natural origin, offers several clinical advantages over other chemotherapies, i.e. broad-spectrum activity, rapid action, low target-based resistance and low immunogenicity [24].

Also known as host defense peptides (HDPs), natural AMPs were first discovered on the external surfaces of amphibians three decades ago [25]. Since then, they have been found from a variety of organisms belonging to all kingdoms of life. Apart from antimicrobial activity, they also may exhibit anticancer, anti-biofilm, spermicidal, or mitogenic activities. By their mode of action, AMPs can be broadly classified into two groups. While one group of HDPs exhibits direct and broad-spectrum antimicrobial activity, the other group modulates the innate immune response of the host [26]. Being very diverse in nature, AMPs have different chemical structures and conformations but with certain common properties, for example, small size (12-50 amino acids long), and either linear or cyclic structures with cationic and hydrophobic sequences [27]. This amphipathic structure enables AMPs to bind to membranes and they have a general mode of action [28,29].

AMPs have been found to act on a variety of pathogens including bacteria, fungi, parasites, and viruses. The majority of AMPs exert their biological activity via: a) membrane disruption or membrane pore formation – such as melittin, LL-37 MSI-78; b) inhibition of cell wall synthesis – such as Class I bacteriocins, nisin, Pep5 etc.; c) inhibition of protein, RNA and DNA synthesis – such as buforin II and pleurocidin [30–35]. Synthetic AMPs (e.g. brilacidin - a mimetic of magainin, currently in phase II) have been developed that selectively damage the microbial membrane [36,37]. Amongst innate immunity modulators, defensins can induce several cytokines such as TNF and IL-1 in monocytes, and IL-8 in lung epithelial cells. Defensins are also potent chemo-attractants for monocytes and neutrophils. For example, LL-37, CRAMP, α-defensins, and β-defensins are known chemo-attractants for monocytes, macrophages, T cells, neutrophils, immature dendritic cells, and mast cells [31,38,39]. AMPs are also known to influence the adaptive immune response by facilitating the uptake of antigen by

monocytes or other antigen-presenting cells. For example, melittin from bees enhances a mixed Th1/Th2 response to tetanus toxoid in mice by promoting IgG and IgG2a antibody production. They can modulate chemokine and cytokine responses depending on their concentration and the order of exposure to cells. They can also facilitate wound healing and angiogenesis. For example, histatin and LL-37 induce fibroblast migration and proliferation [40,41].

There are various databases from which information on naturally occurring peptides can be retrieved online. To date, 17,353 antimicrobial peptide sequences can be retrieved, of which 12,704 are patented peptide sequences [42–51]. The number of such peptides is expected to increase in the future owing to the technological advances in peptide discovery and synthesis, hopefully leading to new antimicrobial drugs with high target affinity and fewer side effects [15].

AMPs are attractive targets regarding human health due to their selective toxicity against bacteria. Some AMPs are extremely target-selective so that very narrow spectrum drugs can be developed, a property that is highly desired since it lowers the chances of emerging and spread of resistances [52]. Currently, AMPs are mainly being studied as single anti-infective agents, but they also are examined in combination with conventional antibiotics or antivirals to promote additive or synergistic effects. A combination could be an immune-stimulatory agent to enhance innate immunity, and an endotoxin-neutralizing agent to prevent septic shock caused by fatal complications of bacterial virulence factors [53]. Table 2 lists some of the AMPs in clinical development.

1.2.2 Anti-cancer peptides

Peptides may exhibit anti-cancer activity through different mechanisms. Earlier peptides for cancer treatment were found by searching for regulatory peptides that target overexpressed G-protein coupled receptors on cancer cells. One of these peptides, somatostatin (a 14-residue cyclic neuropeptide, SST) was found to target five G-protein coupled receptors, one or a few of which are overexpressed in tumor tissues [54]. This led to the development of an SST analog, the disulfide-cross linked octapeptide octreotide, which is being used for the treatment of growth hormone-secreting pituitary adenomas [55]. Later on, multi-receptor binding analogs with higher biostability and affinity were developed, such as pasireotide and somatoprim, which are in clinical trials for octreotide-resistant tumors [56].

Host defense peptides can also have antitumor activity and may specifically target cancer cell membranes [26,59]. Magainin 2 and its derivatives are the very first HDPs investigated for anticancer activity. In animal models, these peptides destroy cancer cells via cell membrane lysis and apoptosis, which is very similar to their antimicrobial action. However, multiple modes of action have been proposed for a single HDP [26,60]. Peptides that target the vasculature supplying nutrients and oxygen to tumor tissue are also being developed. These vessels overexpress a number of receptor targets including adhesion

Table 2. Examples of anti-infective peptides in clinical development [35,57,58]

Peptide	Composition	Intended clinical application	Development stage
Antibacterial peptides			
Omigard	Omiganan pentahydrocholoride	Prevention of intravascular local catheter infection	Phase III
Pexiganan (MSI-78)	Magainin analog	Topical agent for mild diabetic foot infection	Phase III
Iseganan (IB-367)	Synthetic protegin I analog	Oral mucositis, ventilator-associated pneumonia	Phase III
POL7080	Cyclic peptidomimetic	*Pseudomonas* infection	Phase I
Brilacidin (PMX-30063)	Small defensin mimetic	*Staphylococcus aureus* skin infections	Phase II
Lytixar (LTX-109)	Synthetic peptidomimetic	*S. aureus* and *Ps. aeruginosa*	Preclinical
Antifungal peptides			
Novexatin (NP213)	Cyclic cationic peptide	Onychomycosis	Phase II
CZEN-002	Synthetic octapeptide	Vulvovaginal candidiasis	Phase II
PAC-113 (P-113)	12 amino acid histatin derivative	Oral candidiasis	Preclinical
Gomesin	Natural antimicrobial peptide	Systemic candidiasis	Preclinical
Antiviral peptides			
Fuzeon (enfuvirtide)	Synthetic peptide	HIV-infection	Marketed
Tat protein	HIV-1 TAT protein	HIV-1	Preclinical
RhoA (peptide 77-98)	Fragment of RhoA GTPase	Human respiratory syncytial parainfluenza virus-3, HIV-1	Preclinical
LL-37	Human cathelicidin	Influenza A	Preclinical
Mucroporin M-1	Scorpion venom-derived peptide	Hepatitis B virus	Preclinical

molecules – α_v integrins. The best examples of such tumor-targeting peptides are the RGD (Arg-Gly-Asp) and NGR (Asn-Gly-Arg) peptides that are currently in clinical trials [61]. The RGD motif is found in many extracellular matrix proteins and targets $\alpha_v\beta_3$ integrin receptors that are expressed on tumor cells. Consequently, the RGD motif serves as a template for designing peptides that target not only $\alpha_v\beta_3$ integrins but also other integrins expressed during angiogenesis [62]. The NGR peptide, on the other hand, targets aminopeptidase N (CD13), an embedded metalloprotease that is overexpressed in tumor vasculature. The NGR peptide has a high affinity for the active site of the enzyme but is resistant to degradation. Various peptides are under studies that utilize the NGR motif as a selective tool for drug delivery and tumor imaging [63]. A modern approach to delivery is conjugating cancer-targeting peptides with nanoparticles. Such conjugates exhibit highly tunable properties for imaging or killing cancer cells. A recent example is the use of iRGD (CRGDKGPDC) conjugated to carbon nanodots to enhance fluorescence signals

in tumor imaging [64]. Peptides are being engineered with improved immunogenicity, higher specificity in cancer and better mimicking of tumor-associated antigens [65].

Peptides are being investigated as anti-cancer vaccines as well. For example, human papillomavirus (HPV) vaccine for cervical cancers (Gardasil, Gardasil9, and Cervarix) and hepatitis B vaccines for liver cancers have been approved by the FDA [66]. Most of the peptide vaccines focus on the T-cell epitopes, which are able to recognize small peptides, causing a stimulation of the immune system. Several peptide-based cancer vaccines have entered clinical trials, including a vaccine that targets human epidermal growth factor receptor 2 (HER-2) in breast, lung and ovarian cancer [67]. Recently, B-cell epitopes of proto-oncogene proteins were used to develop vaccines, such as the HER-2 vaccine that prevents mammary tumors in transgenic and transplantable mouse models of breast cancer [68]. These vaccines induce immunological memory against cancer-inducing agents and specific cancer cell antigens. Peptide vaccines provide a highly modifiable system but a perfect vaccine that preempts the development of cancer has to be established [69].

The anti-cancer applications hold the largest share in peptide therapeutics and the field is still expanding. With the development of new screening and delivery techniques, the scope for anti-cancer therapeutic peptides will continue to expand [8,69]. Table 3 provides some examples of peptides in clinical trials related to oncology studies.

1.2.3 Peptides for treatment of metabolic and CNS disorders

Most metabolic disorders are related to hormonal malfunction, including growth hormone deficiency, osteoporosis, diabetes, and obesity. Diabetes is the more prevalent disorder and it often brings other associated disorders as well. As peptide and protein hormones play a major role in balancing metabolic processes, the use of peptides and peptide analogs is a very successful therapeutic strategy. For example, insulin is essential for the control of glucose homeostasis, and a deficiency leads to diabetes mellitus. Commercial production of insulin started in 1922 from animal sources. In 1978, insulin was prepared by recombinant technology and approved by the FDA. The first human insulin analog was approved in 1996 as Lispro. In 2006, Exubera, an inhalable insulin, was approved. At this moment, more than 300 human insulin analogs have been identified consisting of 70 animal, 80 chemically modified, and 150 biosynthetic insulins. Modern insulin analogs mostly fall into two categories, *viz*., fast and short-acting insulins that mimic the action of endogenous insulin (bolus insulin, e.g. lispro, glulisine and aspart), and the basal (background) insulin analogs with long-acting profiles (e.g. Glargine) [70].

Table 3. Examples of anti-cancer peptides in clinical development [65,69,71]

Peptide	Description	Phase	Cancer type	Intended use
Imaging agents				
^{18}F-FPPRGD2	^{18}F-labeled pegylated dimeric RGD cell adhesion peptide, targets $\alpha_v\beta_3$ integrins	Phase I/II	Lung cancer, breast cancer, glioblastoma multiforme and other cancers	Imaging agent for assessment of response to antiangiogenesis therapy
^{18}F-Fluciclatide	^{18}F-radiolabeled small peptide containing the RGD cell adhesion moiety	Phase II	Kidney neoplasm	Imaging agent to assess pazopanib systemic therapy
iRGD	Homing RGD cell adhesion peptide	Phase I	Advanced breast and pancreatic cancer	Imaging agent of human cancer
Direct therapeutics				
p28	Azurin-derived cell-penetrating peptide	Phase I	Recurrent progressive CNS tumors	Anticancer agent
Prohibitin-RP01	Regulatory protein	Phase I	Prostate cancer	To treat advanced prostate cancer
GRN1005	Conjugate of angiopep-2 (a peptide facilitating brain penetration) and paclitaxel	Phase II	Breast cancer	To treat breast cancer
Vaccines				
HLA-A*2402 (or HLA-A*0201)	Derived from VEGF-R1 and VEGF-R2 vascular endothelial growth factor mimic	Phase I/II	Advanced solid tumors	Cancer vaccine
StimuVax	Mucin-1 peptide antigen	Phase III	Non-small cell lung cancers	Cancer vaccine
GV1001	Telomerase peptide vaccine	Phase III	Pancreatic cancer	Vaccine for stage III non-small cell lung cancer
SurVaxM	SVN53-67/M57-KLH, survivin peptide mimic	Phase II	Glioma	Newly diagnosed glioblastoma

Recently, glucagon-like peptide-1 (GLP-1) receptor agonists have been developed for Type 2 diabetes (T2DM). These compounds help in diabetes control by the incretin effect of glucose-dependent insulin release, which is mediated by GLP-1. Exenatide, a synthetic version of exendin-4 from the Gila monster lizard, was the first GLP-1 receptor agonist to enter the market. Another GLP-1 agonist, Lixisenatide, was later introduced with prolonged action. Liraglutide and Semaglutide are based on endogenous GLP-1 with subtle modifications in the original peptide chain [72,73]. Recently, glucagon/GLP-1 dual acting (GGDA) hybrid peptides and GLP-1/gastrin dual acting fusion peptides were developed. Peptides such as the GGDA peptide ZP2929 and the GLP-1/gastrin fusion peptide ZP3022 are now in early phase clinical trials [70,74].

Many neuropeptides play a central role in regulating cellular and intercellular physiological responses in the central nervous system (CNS). This suggests they could lead to interesting therapeutic targets for the treatment of CNS disorders. Accordingly, peptides indeed are the forefront leads for the treatment of CNS diseases such as schizophrenia, anxiety, ischemia, degenerative diseases and pain syndromes. Agonists acting in the central nervous system on the neurotensin, cholecystokinin, neuropeptide Y and oxytocin receptors are the major targets for peptide drug development [75–77]. For example, an (AuNP)-LPFFD conjugate radiolabeled with ^{18}F was studied for the diagnosis and treatment of Alzheimer's disease (AD) [78]. Polymer-coupled neuropeptides are also investigated for inhibition of amyloid-β peptides occurring in brain lesions of AD patients [79,80]. Furthermore, the vasoactive intestinal peptide of the glucagon/secretin superfamily and its receptors are interesting leads for treatment of Parkinson's disease [81]. Opioid neuropeptides (e.g. enkephalins, dynorphins, endorphins, nociceptin) acting on G-protein-coupled receptors are considered as potential therapeutics for AD as well. These peptides are involved in the neuroinflammatory components of AD [82–84]. Peptide hormones such as vasopressin and oxytocin are also found to elicit secondary effects that are of interest in the treatment of memory loss and anxiolytic activity [85,86]. Recently, GLP-1 receptor agonists (e.g. exenatide) were shown to improve motor and cognitive function in persons with Parkinson's disease. It is predicted that many of the peptides that are used for the treatment of metabolic disorders could also be used to treat CNS disorders due to the common pathophysiology [87].

Most of the peptide leads for therapy of CNS disorders are in the initial stages of development. Although they show a huge potential for diagnosis and treatment of CNS disorders, the blood-brain barrier (BBB) is a great obstacle. Intranasal delivery of peptide drugs is a potential strategy to bypass the BBB. Nano-carriers for the delivery of peptide drugs and conjugated peptides may facilitate the use of peptides in the treatment of neurodegenerative diseases [82,88].

1.3 Bioactive peptides as food ingredients

Peptides can influence the taste of food. During the last century, peptides, along with amino acids, have been studied for their taste-altering and -enhancing properties. Investigators have characterized peptides as sour, sweet, bitter, savory, or tasteless [89]. The example of the accidental discovery of the famous sweetener aspartame (L-Asp-L-Phe-OMe) is remarkable in this regard [90]. Later, many sweet di-, tri, and tetra- peptides were synthesized. Peptides with bitter taste were also identified in the same time period [91]. So far, peptide hydrolysates from a variety of sources have been extracted and purified. Individual peptides for a characteristic taste have been identified, and to date, this is still an active topic of research [92–94]. There are more than 400 different peptides in the BIOPEP database of plant-derived products, only from food-related sources, with

experimentally determined sensory stimulation [95]. These peptides are very interesting to the industry for marketing food products with unique and enhanced tastes.

Dietary proteins contain specific amino acid sequences that are partially released by the action of proteases in the gut or by the activity of microbial enzymes. Most of the bioactive peptides are between 3 to 20 amino acids in length and their activity depends on the amino acid sequence and composition [96]. Taste-promoting peptides show very diverse composition and properties[4,97]. Many peptides are also used as food additives because of health benefits. Such bioactive peptides can produce, for example, antihypertensive, antithrombotic, opioid agonist or antagonist, immunomodulatory, anticancer, antimicrobial and antioxidant responses[98,99]. The market for such food products containing bioactive peptides is growing due to the increasing consumer awareness about the possible health benefits of such functional foods and nutraceuticals [100–102].

Antihypertensive peptides are one of the most extensively studied groups of bioactive peptides with many of them commercially available. These peptides inhibit angiotensin-converting enzyme (ACE) and renin; thus, help to maintain the normal blood pressure and prevent hypertension. A large number of ACE inhibitory peptides have been identified in snake venoms, digested food proteins, and especially milk. Examples are the Val-Pro-Pro and Ile-Pro-Pro tripeptides that are hypotensive and immunomodulatory components released by β-casein and κ-casein upon enzymatic digestion [103,104]. Much less information is available regarding bioactive peptides that inhibit the angiotensinogenase activity of renin. However, a recent example is the peptide Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala from the red seaweed *Palmaria palmate*. This peptide was shown to reduce the blood pressure in spontaneously hypertensive rats by inhibiting renin activity *in vivo* [105].

Another important class of food peptides is the antioxidant peptides that reduce oxidative stress in the human body or prevent oxidative conversions during preparation and storage of foods. In the body, such peptides can prevent harmful effects of free radicals that are formed as a result of oxidative processes. The health benefits of antioxidant peptides are manifold and they can play an important role in the prevention and treatment of chronic degenerative diseases such cardiovascular diseases, cancer, rheumatoid arthritis or diabetes [106]. Consequently, such peptides are attractive as food additives. Peptide fractions (either purified or as protein hydrolysates) with antioxidant activity can also be added to food products to reduce oxidative changes during storage. The effect has been found superior to that of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which pose potential health issues [107]. Antioxidant peptides can be used as food emulsifiers to reduce lipid peroxidation in emulsion-type food products [108]. Peptides with antioxidant activity have been found in hydrolysates of plant and animal proteins, as well as in peptide fractions

from mushrooms, algae and marine species that can be incorporated in foodstuffs [106,109–111].

Immunomodulating peptides occurring in or derived from food components have been found to stimulate the proliferation of macrophages into lymphocytes and phagocytes, or in antibody synthesis and cytokine regulation, such as Oryzatensin (GYPMYPLPR) from rice albumin [112]. Fermentation of milk releases ACE-inhibiting peptides including the αS1-immunocasokinin (TTMPLW) peptide from α_{s1}-casein (ft194-199) that shows immunomodulatory activity. The peptide was shown to stimulate phagocytosis of sheep red blood cells by murine peritoneal macrophages [113]. Other immunopeptides from β- and κ-casein, as well as from α-lactalbumin are also mentioned in the literature [114]. Antithrombic peptides inhibit the aggregation of platelets and fibrinogen activation. Casopiastrin (ft106-116), a member of κ-casein–derived casoplatelin peptides, inhibits the fibrinogen-binding process [115]. Pepsin digests of human and sheep lactoferrin also inhibit thrombin-induced platelet aggregation. Such peptides are recommended for the prevention of thrombosis occurring in patients affected by coronary heart disease or other blood system diseases [116,117]. Casein phosphopeptides can function as mineral carriers by forming soluble organophosphates. They serve in calcium absorption under physiological conditions and inhibit caries lesions via recalcification of the dental enamel, making them attractive targets for dental therapy [118]. Recently, bioactive peptides were studied for chemoprevention, i.e. to prevent carcinogenesis. Anti-cancer peptides have been discussed in previous sections. The use of nutraceuticals as anticancer peptides to reduce the risk of cancer is being investigated but limited data are available from human trials [119,120]. Table 4 lists some of the prominent peptide products marketed by the food industry.

1.4 Cosmeceuticals (cosmetic peptides)

Cosmeceuticals are products that are applied topically to boost the skin appearance. Cosmeceuticals containing biologically active ingredients are considered a combination of cosmetics and pharmaceuticals, imparting therapeutic, disease-fighting or healing properties to the skin cellular structure. These products can come in the form of creams, lotions, serums, and ointments [125]. The cosmetic industry has been using peptides in cosmetic products for around 20 years owing to their anti-aging properties [126]. Nowadays, peptides are an important part of the products of this industry. The increasing evidence for various beneficial effects of peptides is driving the cosmetics industry to introduce an even larger number of peptide-based products in the market. Various studies have shown that peptides can perform many functions including modulation of cell and tissue inflammation, stimulation of collagen synthesis, control of angiogenesis and melanogenesis, and modulation of cell proliferation and cell migration. The high effectivity is also due to the penetrating ability of peptides through the upper

Table 4. Examples of bioactive peptides identified from food sources [121–124]

Product name	Substrate /Source	Product type	Peptide sequence	Bioactivity
Ameal S 120, Ameal S, Evolus, Calpis	Milk	Sour Milk, Beverage, Tablet	IPP and VPP	Antihypertensive
Casein DP, Perptio Drink, C12 Peption	Milk	Beverage, Ingredient	FFVAPFPEVFGK	Antihypertensive
Goma Papucha	Sesame	Beverage	LVY	Antihypertensive
StayBalance RJ	Royal jelly	Beverage	VY, IY, IVY	Antihypertensive
Peptide Nori S, Mainichi Kaisai	Seaweed (*Porphyra yezoensis*)	Beverage, Powder	AKYSY	Antihypertensive
Lapis Support, Valtyron	Sardine	Beverage, Ingredient	VY	Antihypertensive
PeptACE, Vasotensin, Levenorm, Peptide ACE 3000, Peptide tea	Bonito	Capsules, Tablet, Powder	LKPNM	Antihypertensive
Seishou-sabou	Blood (bovine, porcine)	Beverage	VVYP	Weight management
Remake CholesterolBlock	Soy	Beverage	CSPHP	Cholesterol-lowering
Lactium	Milk	Beverage, Capsules	YLGYLEQLLR	Stress relief
ProDiet F200	Milk	Tablets, Capsules	α_{s1}-CN(f91-100)	Stress relief
Antistress 24	Fish	Capsules	NA	Stress relief
PeptiBal	Shark	Capsules	NA	Immunomodulatory
Glutamin peptide, WGE80GPA, WGE80GPN, WGE80GPU	Gluten	Dry milk protein hydrolysates	Glutamine-rich peptides	Immunomodulatory
Capolac, Tekkotsu Inryou, Kotsu Kotsu calcium, CE90CPP	Milk	Ingredient	CPP	Helps mineral absorption
Recaldent	Casein	Ingredient, Chewing gum	SPPQQ as cluster sequence	Remineralization of tooth plaques
BioPURE-GMP	Whey	Ingredient	Glycomacropeptide	Anticarcinogenic, antimicrobial, antithrombotic

layer of the skin and its capability to support and upregulate collagen synthesis [126,127]. Cosmeceutical peptides are generally classified into three types based on the mechanisms of action: signal peptides, carrier peptides, and neurotransmitter-inhibiting peptides.

Recently, there is growing interest in signal peptides which act as messengers to trigger fibroblasts to synthesize collagen or decrease the collagenase-mediated breakdown of existing collagen. This results in firmer and younger-looking skin and studies indicate beneficial effects on wound healing as well [128–130]. Apart from enhancing collagen synthesis, some peptides also modulate elastin synthesis, for example, palmitoyl oligopeptide (palmitoyl-VGVAPG) [131]. Peptides can also modulate melanin synthesis in melanocytes, either by stimulating or by inhibiting melanin production (for tanning or lightening). An example is decapeptide-12 (YRSRKYSSWY) that reduces melanogenesis by inhibiting tyrosinase in human melanocytes [132]. Other effects may be stimulation of lipolysis for trimming or induction of soothing effects. Hair growth stimulation or hair loss prevention can be affected by peptides such as biotinoyl-Gly-His-Lys [133].

Carrier peptides stabilize and deliver important trace elements for wound healing and enzymatic processes. Copper is a very important element for angiogenesis, wound healing, and many enzymatic processes. Peptides can be used to deliver and stabilize copper for these processes. Some of the copper enzymes, like lysyl oxidases, are involved in the synthesis of collagen and elastin, giving improved skin texture and appearance. Superoxidase dismutase eliminates free radicals, reducing their aging effects. Cytochrome c oxidase is involved in mitochondrial energy production and decreasing oxidation of endothelial cells and so improving blood flow to the skin. The carrier tripeptide GHK, which also acts as a signaling peptide, facilitates the uptake of copper by cells, resulting in: 1) increased levels of metalloproteinase 1 and 2 and thus contributing to the remodeling of aging skin; 2) stimulation of type 1 collagen and certain glycosaminoglycans (dermatan sulfate and heparin sulfate); 3) facilitation of the enzymes lysyl oxidase and superoxide dismutase, as well as of cytochrome c. The combined effects result in improved skin firmness and texture, and in diminished wrinkles, fine lines, and hyperpigmentation.

Peptides that inhibit neurotransmitter activity are the most recent class of cosmeceuticals. These peptides were developed to mimic botulinum neurotoxins; they block acetylcholine release at the neuromuscular junction. They reduce facial muscle contractions, thus softening wrinkles and fine lines. Peptides may inhibit enzyme activity either directly or indirectly, producing cosmetic effects in both cases. This includes a variety of peptide mixtures from soy, rice and silk fibroin. Oligopeptide mixtures from rice inhibit matrix metalloproteinase. These peptides also affect human keratinocytes by stimulating hyaluronan synthase 2 expression [134]. Soy peptides inhibit proteinases, p53

expression and Bax protein expression in epidermal cells that were exposed to UV-B irradiation [135]. Table 5 lists some of the important peptides used as cosmeceuticals.

Table 5. Examples of peptides used in cosmetics

Peptide	Bioactivity	Commercial name
Signal peptides		
VGVAPG [139–141]	Stimulates collagen synthesis, chemo-attracts fibroblasts for matrix repair, downregulates elastin expression.	
Palmitic acid – KVK [142]	Binds latent TGF-β and induces fully functional TFG-β resulting in collagen production, inhibits MMP.	Palmitoyl tripeptide-5 Syn-COLL
Palmitoyl-VGVAPG (palmitoyl oligo-peptide) [143,144]	Same as palmitoyl-KVK. Enhanced penetration of the epidermis. Repairs age-related skin damage.	Biopeptide-EL (Dermaxyl)
KTTKS[145]	Positive feedback effect on collagen (type I and type II) synthesis as well as fibronectin.	
Palmitoyl-KTTKS (palmitoyl penta-peptide-4) [137]	Enhanced penetration.	Matrixyl Pentapeptide-4
Pal-GHK, Pal-GQPR [146,147]	Supports activation of cutaneous tissue repair.	Matrixyl 3000
GHK / Palmitoyl-GHK (also a carrier peptide) [148,149]	Increases collagen and glycosaminoglycans (GAG) production by stimulating fibroblasts.	Biopeptide-CL
YYRADDA [150]	Inhibits procollagen C-proteinase that cleaves C-propeptide from type I procollagen. Results in decrease in collagen breakdown.	
Elaidyl-KFK [151]	Activates latent transforming growth factor-beta (TGF-β) via the peptide domain, resulting in increased collagen levels and reduced collagenase levels.	Lipospondine
FVAPFP [152]	Mechanism unknown, upregulates the gene expression of extracellular matrix as well as others related to cell stress.	Peptamide 6 Hexapeptide-11
YRSRKYSSWY (Decapeptide-12) [132,153]	Inhibition of tyrosinase. Reduces melanogenesis.	Lumixyl
Neurotransmitter-inhibitors		
Acetyl-EEMQRR-NH_2 [154,155]	Competes with SNAP-25 in the SNARE complex thereby inhibiting release of neurotransmitters.	Argireline (acetyl hexapeptide-8)
Pentapeptide-18 [156,157]	Blocks the calcium channels by coupling to the enkephalin receptor in the neurons inhibiting acetylcholine release.	Leuphasyl
beta-AP-Dab-NH-benzyl·2AcOH [158]	Reversible tripeptide antagonist of muscular nicotinic acetylcholine receptors (mnAChR) at the postsynaptic membrane.	Syn-Ake tripeptide-3
GPRPA-NH_2 [159]	Blocks the acetylcholine postsynaptic membrane receptor.	Vialox pentapeptide-3

In conclusion, peptides are very important constituents of cosmetics. They possess great potential for cosmetic use because of their explicit activities. Most of the advertised products claim to slow or even reverse skin aging. Peptides can do this in multiple ways, either by stimulation of collagen synthesis or through inhibition of neurotransmitter release, both reducing fine lines caused by facial muscles contractions [127]. Like other peptides, cosmeceutical peptides also have some caveats. Some high molecular weight peptides are unable to penetrate the stratum corneum, the outermost dead cell layer of the skin [136]. Natural oligopeptides, therefore, do not easily reach their targets. Palmitoylation of short peptides has solved this problem to a great extent [137]. Other factors, like peptide solubility, bioavailability, potential toxicity problems, biostability, biodegradability, and functional compatibility with the formulations need to be considered. Furthermore, most of the studies that recommend peptide additives in cosmetics are done *in vitro*, which might not always be reproduced under *in vivo* conditions [138].

1.5 Peptide Sources

Peptides can be obtained in a variety of ways. They can be directly isolated from natural sources (e.g. plants, humans, animals, insects, and other organisms like bacteria and fungi), be produced by recombinant synthesis, or be obtained by chemical synthesis, which can also be used to produce peptide libraries [1,15,18,160–163]. Important examples of biological sources are the saliva of a lizard (Gila monster – Exenatide) and viper venom (Lancehead viper – Captopril). Plant or fungal toxins have also proved to be a fruitful source of peptides and stable peptide scaffolds that can serve as a framework to add an active molecule graft, for example, SFTI-1, a trypsin inhibitor from sunflower [15,164]. Plectasin (later modified to NZ2114) was discovered from a cDNA expression library of a mushroom fungus (*Pseudoplectania nigrella*)52. The microbicidal and spermicidal peptide sarcotoxin Pd was isolated from rove beetles (*Paederus dermatitis*)[165]. Peptides have been isolated from sea creatures, such as mitomycin that was isolated from blue mussel *Mytilus galloprovincialis* [166]. A whole class of lasso peptides with a variety of medically interesting functions, of which antimicrobial activity is the most common, has been identified in bacteria [167].

The use of natural resources can be advantageous for the discovery of bioactive peptides. Interesting peptides can be detected directly via pharmacological screening and extraction from a material like an animal venom that should have bioactive components. Genomic information can also be useful, and a combined approach can lead to a specific peptide of interest [15]. For example, in the CONCO project peptide XEP-018 (a painkiller and local anesthetic) was discovered using genome mapping, transcriptome analysis, and proteomics of a venomous snail (http://conco.ebc.ee/) [168]. Natural sources, however, have not proven to be economically viable for production of pure peptides in

large quantities. Nisin, a lantibiotic, is an exception; it is a natural fermentation product of *Lactococcus lactis*. Chemical synthesis and recombinant DNA technology are the methods of choice for large-scale production of peptides [169].

Many bioactive peptide sequences occur in inactive form while embedded in the sequence of a larger protein. They are released from their parent molecule by proteolytic cleavage, e.g. during microbial fermentation of proteins or during proteolysis by gastrointestinal enzymes. Bioactive peptides are abundantly found in milk and dairy proteins where they are encrypted in the caseins or whey proteins. Consequently, they are also found in dairy (by-)products. In recent years bioactive peptides produced by marine organisms are also receiving a lot of attention, e.g. for use as nutraceuticals. Bioactive peptides extracted from fish, sponges, ascidians, seaweeds, and mollusks are potent food additives because of their antihypertensive, antioxidant, and antimicrobial characteristics.

As high amounts of a bioactive peptide must be added to food to produce a desired biological effect, large-scale production is in demand. For industrial production in the food industry, microbial processes and proteolytic release from plants and microbes are favorable. The food industry utilizes proteolytic starter cultures and/or gastrointestinal enzymes to cleave whole proteins to release bioactive peptides [4]. Chemical processing of food proteins with acid, alkali, heat and enzymatic hydrolysis can also release bioactive peptides [111]. Peptides can also be expressed in algae as chimeric proteins, which upon digestion release smaller bioactive peptides [170]. In addition to natural sources, peptides can also be designed and synthesized in a lab. Peptides designed *in silico* can serve as a huge resource for new lead peptides with better stability, bioavailability, and higher yields [4,171].

1.6 Limitations to peptide applicability

As mentioned in previous sections, peptides have great potential in a wide array of applications. However, their use may suffer from certain disadvantages. Therapeutic peptides, especially AMPs, may exert undesirable toxicity in humans. Cationic AMPs usually target negatively charged lipopolysaccharides present in the bacterial cell membrane. Due to the presence of zwitterionic lipids in the human cell membranes, some AMPS may cross-target human cell membranes as well as extracellular surfaces resulting in unwanted toxicity [172]. Most peptides are poorly absorbed from the gut into the bloodstream. Another limitation can be the low stability of peptides under physiological conditions [173]. They are potential targets for serum proteases, which reduces bioavailability and *in vivo* stability. The structural stability can be affected by a number of factors such as salt and pH that may, however, hinder in vivo activity [173]. Furthermore, the production cost at the moment is very high compared to small chemical drugs. There is also a lack of commercially viable processes (e.g. chromatographic and membrane

separation techniques) for large-scale downstream processing [4,174]. Further studies on chemical modifications, use and synthesis of hybrid peptides, conjugative formulations, use of unnatural amino acids, and resistance to proteases are anticipated to improve the application potential of therapeutic peptides. In parallel, mechanisms of peptide activity need to be further elucidated that will help to design better peptides for improved absorption and pharmacokinetics and reduce the risk of undesired immune responses [4,175].

Part II. Peptide synthesis

Many useful bioactive peptides can be obtained from natural resources. However, the amounts that can be isolated often do not satisfy the increasing demand of industries which aim to utilize peptides in commercial products. The isolation of peptides from natural sources often is cumbersome in terms of availability of raw material and the yield of the final purified peptide is insufficient in most cases [4]. This drives the need for synthetic approaches for peptide production, and strategies that are cost-efficient and quick are in high demand [1]. Chemical peptide synthesis can also provide structures that deviate from nature's repertoire and may include molecules containing non-proteinogenic, unnatural or chemically modified amino acids, which may be important for research as well as in therapeutic application. Synthetic strategies can impart modifications such as hydroxylation, phosphorylation, sulfation, glycosylation, and disulfide bond formation. It may also be used for reactions such as methylation and cyclisation, and for preparation of retro–inverso peptides in which the amino acid sequence is reversed and the α-carbon chirality of the amino acid is inverted. Preparation of thiopeptides and peptides with iso-peptide bonds as well as the introduction of non-α-amino acids are also possible [2].

Industrial production of peptides can be achieved biologically (via fermentation, non-ribosomal expression, and cell-free expression), chemically (solid- or solution-phase synthesis), or chemo-enzymatically. The size of the target peptide often determines which route to follow. We will elucidate these methods in detail in the next sections.

2.1 Biosynthesis

Fermentative peptide synthesis utilizes the natural machinery of a cell to produce peptides. This is the cheapest and most environment-friendly way to produce peptides and proteins. Fermentation is certainly the method of choice for the synthesis of large peptides. The major advantage is the regio- and stereo-specificity of the obtained product. Development of a peptide synthesis process by fermentation involves gene synthesis or isolation, expression of the recombinant DNA in a suitable host, cultivation, and downstream processing [2]. Fig. 1 gives a general overview of the steps involved during fermentative production of a peptide via recombinant DNA technology.

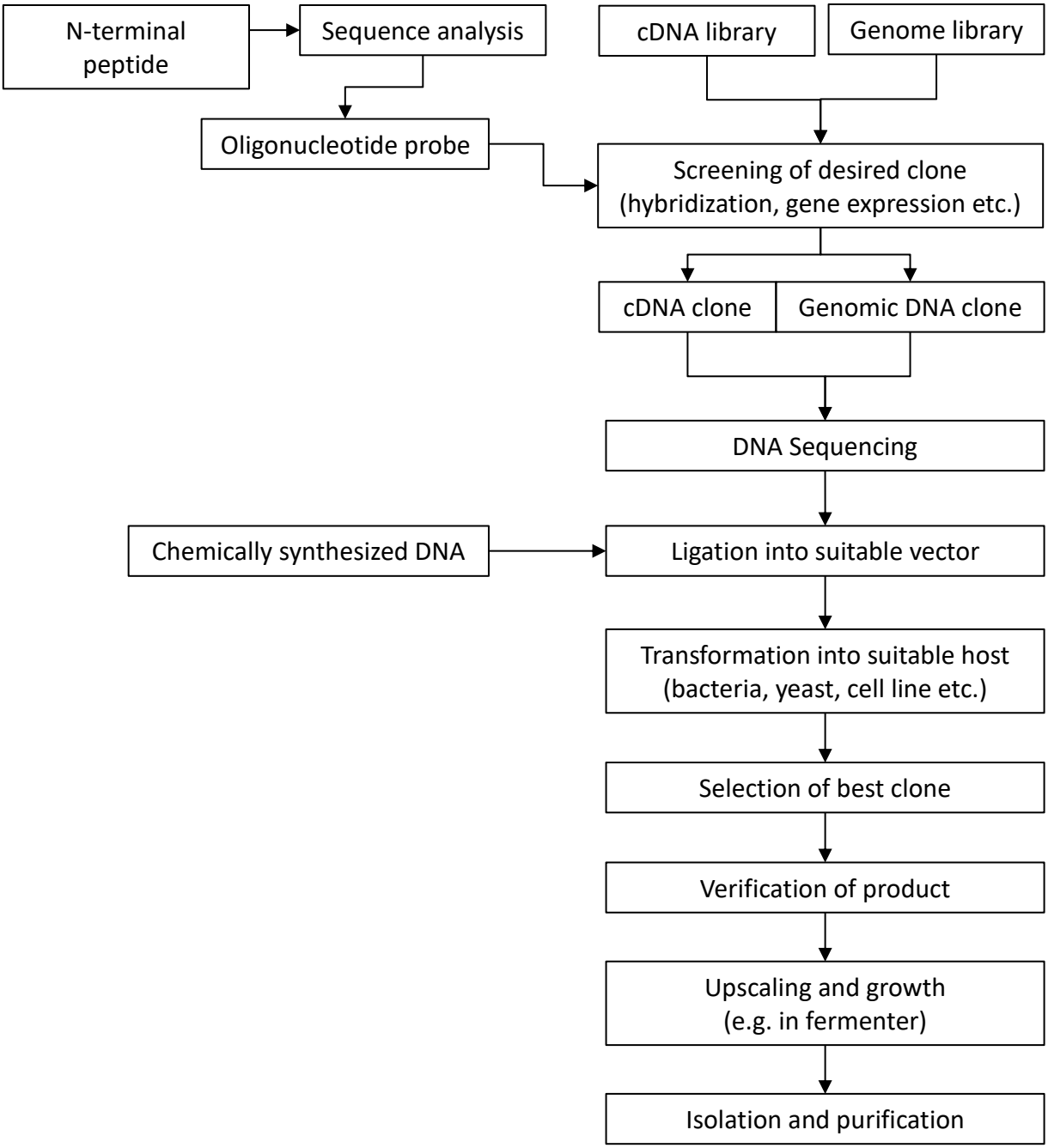


***Fig. 1**. Overview of steps for recombinant peptide/protein production [2]*

The encoding DNA can be of many origins, either amplified from genomic DNA or using cDNA synthesized from mRNA, or it can be obtained by de novo chemical synthesis. Often, the DNA fragment needs to be codon-optimized to match the preference of the expression host [176]. Selection of the host system is a major task. The first choice is most often *E. coli*, which in fact suffers from many drawbacks. It lacks the enzymatic machinery for many post-translational modifications (e.g. glycosylation) and efficient peptide secretion [177]. It also contains endotoxins that might enter into the

purified peptide and make it difficult for a safe application [178]. To overcome these obstacles, eukaryotic systems like yeast and mammalian expression systems can be utilized [179]. Such systems for fermentative peptide synthesis can produce large amounts of recombinant peptides but need to be optimized for every single peptide/protein and for each host expression system. This task is labor-intensive and becomes more complicated in case of larger expressed peptides. Peptides on the smaller end of the size spectrum tend to form insoluble particles and cellular proteases can degrade them, decreasing the product yield [180]. It is also difficult to introduce non-proteinogenic amino acids into peptides [181]. Peptides might not always be secreted by the expressing organism which often leads to complicated purification strategies [182,183].

Even though the limitations of fermentative peptide synthesis seem daunting, numerous peptides of pharmacological interest have been synthesized this way. Historically, somatostatin is the first example of a peptide produced by recombinant DNA technology (1977). The DNA for this peptide hormone was chemically synthesized, codon optimized for *E. coli*, and inserted into a suitable expression plasmid as a fused protein with β-galactosidase to prevent degradation by cellular proteases. The peptide was later cleaved from β-galactosidase by the action of cyanogen bromide to release the mature hormone [184]. About a year later, recombinant production of human insulin was reported. The two chains were expressed separately in two different bacterial strains and later joined by disulfide bonds [185]. Still later, human fibroblast interferon (F-IF) was cloned from cDNA, which is devoid of introns common to eukaryotic systems, so expression in *E. coli* was relatively straightforward [186]. Codon optimization of synthetic DNA was used to improve the production of INF-β [187]. The production of human growth hormone is also worth mentioning here, as the gene could not be expressed straightforwardly in a bacterial system since the gene contains many introns. Furthermore, it contains a signal sequence needed for export out of the cell that must be cleaved off to release the mature hormone. Cloning and expression from cDNA initially gave a suboptimal peptide yield (2.4 mg/L)[188]. The system was later optimized by fusion to the *E. coli ompA* signal sequence, which resulted in the export of the peptide to the bacterial periplasm, thereby enabling disulfide bond formation [189]. Similarly, interferon-β production yield was also improved by directing the peptide to the periplasm [190]. Production of insulin was also optimized by expressing as a fusion peptide such as proinsulin that was subsequently transformed into active insulin by enzymatic cleavage [191]. Other tags such as maltose-binding protein (MBP), bacterial transcription factor NusA, glutathione-S-transferase (GST), solubility-enhancing tag (SET), and small ubiquitin-related modifier (SUMO) etc. have been used to obtain better expression of difficult peptides as well [192]. However, with high expression levels, inclusion bodies tend to form, reducing the activity of the peptide due to misfolding. This problem can also be solved in many ways. For example, by expressing peptides as concatamers, as in the case

of proinsulin, neuropeptide substance P, and the self-assembling peptide P_{II}-2, which were later cleaved to release individual peptides [193–195]. Other expression systems including baculovirus-infected insect cells, yeast cells, transgenic tobacco plant cells, and *in vitro* cultured tracheal epithelial cells have also been developed to express difficult peptides [196,197].

2.1.1 Non-ribosomal peptide synthesis

Another method for utilizing the microbial cell machinery to synthesize peptides is the use of non-ribosomal peptide synthesis (NRPS). In this approach, one utilizes bacterial multimeric enzyme complexes that work without mRNA and ribosomes. Instead, the process makes use of multifunctional modular NRP synthetases that contain distinct functional domains, for example, adenylation, thiolation, and condensation domains. The whole system for the incorporation of an amino acid is called a 'module'. The connected modules serve as the template as well as the biosynthetic machinery. These synthetases create peptides that may have unique structural properties including rigidity (via cyclization or oxidative cross-linking of side chains) and high stability. The inclusion of D-amino acids, fatty acids, glycosylated amino acids, *N*-methylated amino acids, *N*/*C* terminally modified amino acids, and heterocyclic rings is also possible. Microbes use NRPSs to synthesize secondary metabolites that are often crucial to the organisms' survival. Both bacteria and filamentous fungi use this machinery to produce antibiotics and other bioactive compounds that may be used as therapeutics. Examples are bacitracin, actinomycin, gramicidin S, surfactin, and precursors of β-lactam antibiotics [2,198,199]. After successful fermentative production of the cyclic dipeptide D-FP-diketopiperazine in the heterologous host *E. coli*, there has been a lot of attention toward utilizing this tool for peptide synthesis. Recently, antitumor peptides (echinomycin and triostin A) were totally synthesized utilizing a recombinant NRPS machinery in *E. coli* [200]. The system allows for the one-pot synthesis of complex peptides from simple carbon and nitrogen sources. The NRPS module can be fine-tuned for biosynthesis of novel peptide molecules, by swapping individual domains in the module from other NRPS complexes, deleting or inserting of certain modules, or by introducing mutations in a different domain of the module by protein engineering [201,202]. In practice, however, this is a complicated task due to delicate interactions between the domains that must be maintained for proper peptide synthesis. Nevertheless, the last decade has seen notable progress in the engineering of novel NRP products. One bottleneck is that each synthetase module has to be fine-tuned for the formation of a single substrate-specific peptide bond. As the technologies are advancing, mutants can be generated more quickly and larger numbers of variants can be tested. Predictions with computational tools are very promising in this regard. Engineering of NRPSs thus may become increasingly important for the generation of small peptides as natural product analogs [203].

2.1.2 Cell-free expression systems

As mentioned earlier, production of short peptides by fermentation is problematic. Even after an overexpression system is developed, the desired peptide can aggregate and form inclusion bodies that may hamper growth or the peptide aggregates may be too unstable to carry on for downstream processing. For such reasons, as outlined above, fermentation is most promising for large peptides (50 to >100 amino acids). Many of the problems could potentially be avoided by using *in vitro* cell-free translation systems, which are commercially available [204]. Continuous-flow cell-free systems (CFCF) and continuous-exchange cell-free systems (CECF) have been developed with improved peptide turnout [205]. These systems grant addition of amino acids, ATP, and GTP continuously to the reaction mixture and tandem removal of the peptide product. This was demonstrated by producing cecropin P1 (a mammalian AMP consisting of 31 amino acids) fused with green fluorescent protein (GFP), albeit with low yields reported [206]. Many modifications based on the original cell-free transcription-translation system have been developed, either to obtain higher yields or to increase the capacity of the system. Microfluidic array devices with cell-free expression of peptides have been developed and used for the successful synthesis of GFP and luciferase. Such a device could be applied for high-throughput expression, and although not suitable for large quantities, it would be possible to prepare arrays of different peptides in a short time. Furthermore, unnatural and chemically modified amino acids could be incorporated, and protein folding could be optimized by modification of reaction conditions [205,207]. A recent example is the WGCFS (wheat germ cell-free expression system) that introduced high-level multiplexing. The system has a potential of producing tens of thousands of peptides in few weeks [208].

2.2 Chemical synthesis

Chemical synthesis is considered as the best method for the synthesis of small to medium-sized peptides (5 – 50 amino acids). It is by far the most mature technology for peptide synthesis and can be used to synthesize most possible peptide sequences, including many that are difficult to express in bacteria and peptides that contain unnatural amino acids [1,209]. Chemical peptide synthesis methods generally utilize two general steps. First, protecting groups for amino acids are selected, along with a deprotection and activation reactions. Second, a peptide bond is formed between the two building blocks [210]. The protecting groups are selected such that the amino acid side chains do not undergo peptide bond formation; peptide bonds are only formed between an amino acid (or peptide) that is protected at its amino group (or *N*-terminus) and an amino acid (or peptide) that is protected at its carboxylate group (or *C*-terminus). This prevents polymerization of the same amino acids (or peptides) after activation. After the coupling step, protection at the amino group is removed so that it becomes available for

next coupling step. When the desired peptide is synthesized, selective removal of the amino acid side chain protecting groups is done to obtain the final product [1]. The protecting groups are preferably orthogonal so that the deprotection at either *C* or *N* termini occurs independently of each other and does not interfere with the side chain protecting groups, which are usually removed after the last step of synthesis [210,211]. Chemical peptide synthesis is usually achieved via three routes i.e. solution phase peptide synthesis, solid-phase peptide synthesis, and a hybrid approach.

2.2.1 Solution-phase peptide synthesis

Solution-phase peptide synthesis (SPS) is the method of choice when the required peptide length is less than 15 amino acids [212]. It is mostly done in a stepwise manner. An *N*-protected amino acid is coupled with a *C*-protected amino acid, giving an *N*- and *C*-protected dipeptide. Depending on the strategy adopted, further coupling can be carried out in two ways. In case of synthesis in the *N*→*C* direction, the *N*-terminus is kept protected and *C*-terminus is deprotected and activated to be available for next condensation reaction with another *C*-protected amino acid. In case of synthesis in the *C*→*N* direction, the *C*-terminus would be kept protected and the *N*-terminus would be deprotected to react in the next step [213]. The choice for coupling in the *N*→*C* or *C*→*N* direction is mainly based on the cost of the protecting groups, which must be added to each amino acid that needs to be incorporated and must be removed after each peptide bond formation step. The *C*-terminal protected amino acids (e.g. as carboxamide or *tert*-butyl (^{t}Bu) ester) are cheaper, thus making *N*→*C* synthesis the preferred choice in most cases [2,214]. The side-chain protecting groups are removed after the last coupling step and the product is purified. Fig. 2 shows a schematic representation of a solution-phase peptide synthesis strategy.

Solution-phase peptide synthesis is a very flexible approach to peptide synthesis with the additional advantage that peptides and intermediate products can be isolated and purified at each step and further steps can be designed at ease [1]. The process can be repeated in an iterative manner to expand the peptide chain, but it is also possible to prepare protected fragments that are coupled together to obtain large peptides. In principle, the process should give high yields and allow for rapid synthesis, but in practice, partial racemization and formation of by-products may cause problems. In some cases, racemization could be avoided e.g. in case of oxazolone formation during activation and coupling of the carboxyl group [210,215]. Racemization is avoided when coupling involves glycine or proline. Proline resists racemization because of its ring structure [215]. Besides the high purity of the final product, the necessary purification of intermediates after every coupling step can make the process considerably time-consuming in case of long peptides. The use of protecting groups also adds to the costs of the process [1]. The growing peptide chain, containing fully protected amino acids, tends to be poorly soluble even in the presence of organic solvents. This can make the process

slow and cause incomplete conversion resulting in by-products [216]. Also, the process utilizes toxic organic solvents (e.g. diethyl ether, trifluoroacetic acid (TFA), hydrofluoric acid (HF), and hydrazoic acid), which is not environment-friendly. These phenomena limit the feasibility of solution-phase peptide synthesis to short peptides [1,2].

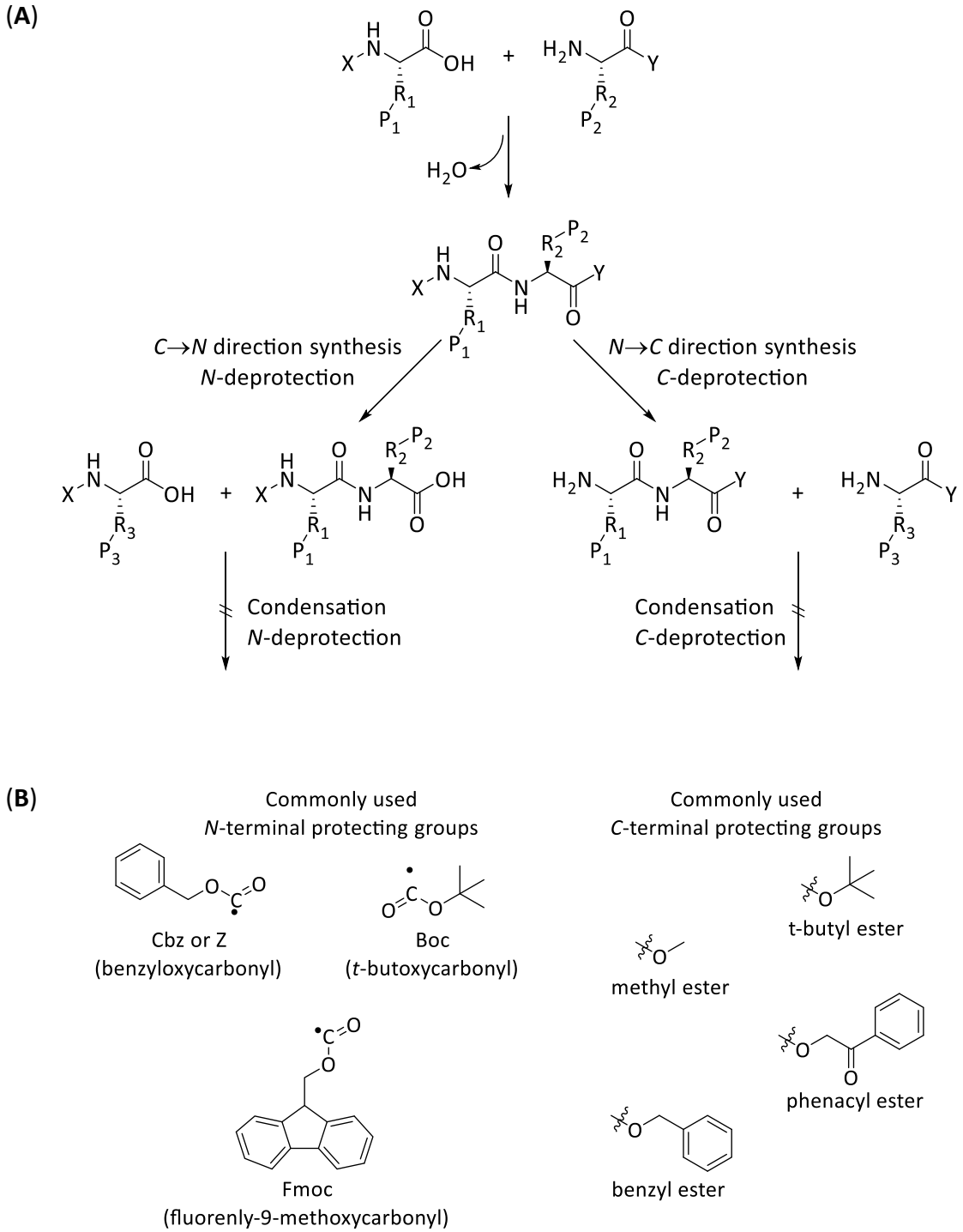


***Fig. 2**. (**A**) Solution phase peptide synthesis (R = amino acid side chain, X = N-terminal protecting group, Y = C-terminal protecting group, P = side chain protecting group (if present)). (**B**) Examples of commonly used N-terminal and C-terminal protecting groups.*

2.2.2 Solid phase peptide synthesis

Solid-phase peptide synthesis (SPPS) was introduced by Merrifield in 1963 by synthesizing the tetrapeptide Leu-Ala-Gly-Val [217]. In SPPS, the first amino acid of the desired peptide chain is attached via its carboxylic acid group to a polymeric support. Subsequent coupling with an α-amino protected amino acid results in the formation of a support-bound dipeptide. In the next step, the α-amino group from the dipeptide is deprotected and coupled to the next protected amino acid. The coupling and deprotection cycle is repeated until the desired peptide chain is achieved. The desired peptide is obtained by removing all the protecting groups from the chain and cleaving the peptide from the polymeric support [1,2]. Fig. 3 shows a schematic representation of SPPS.

The process has many advantages compared to solution-phase peptide synthesis. SPSS allows the reaction systems to be automated and it excludes the issue of peptide solubility as the growing peptide chain is attached to a solid polymeric support. The process is much faster, cheaper to develop, and requires fewer solvents [212,217]. It also excludes the necessity of tedious isolation and purification of the intermediates. Therefore, SPPS is the method of choice for synthesizing medium to large peptides (10 - 50 amino acids). As the growing peptide chain stays bound to the polymeric support, the product can be easily obtained at the end and any by-products or excess reagents can be removed by filtration. The concept of solid-phase synthesis has found application in other fields requiring iterative synthetic steps, e.g. in oligonucleotide synthesis.

The original SPPS protocol utilized *t*-butoxycarbonyl (*t*-Boc)/benzyl combination as the protecting groups. *t*-Boc is used for the protection of the α-amino group and the benzyl ester for the side chains of several amino acids. The method utilizes trifluoroacetic acid (TFA) and hydrofluoric acid (HF) for cleavage of *N*-terminal *t*-Boc groups and for side-chain deprotection, respectively. This method is still widely used for specialist applications. Later introduction of the alternative protecting 9-fluorenylmethoxycarbonyl (Fmoc)/benzyl combination has increased the versatility of SPSS [218]. Today, Fmoc SPPS is the most preferred method of choice in the industry because of the mild deprotection and cleavage possibilities.

Despite its wide use, SPPS has still to achieve its full potential. Although the method has the advantage of using fewer reagents than solution-phase peptide synthesis, lower chances of racemization, and faster turnout time, there is a need for constant improvement of side-chain protection strategies in order to obtain pure peptide products that are free from any side products. Most of the reagents are costly and used in large excess, especially in the early stages of development at the lab scale. Inter-chain aggregation (hydrophobic collapse) may occur in regions where apolar interactions (e.g. between protected side-chains or hydrophobic groups) can lead to a gel structure, making it difficult to proceed beyond the intermediate steps. This can result in problems

during purification and cause a low yield, or even give production of truncated forms of the target peptide [219]. In such a case, peptide synthesis often cannot proceed over 10-15 amino acids. Furthermore, after solid-phase synthesis, the peptides still have to be purified by HPLC, which adds to the production cost. The length limit of peptide synthe-

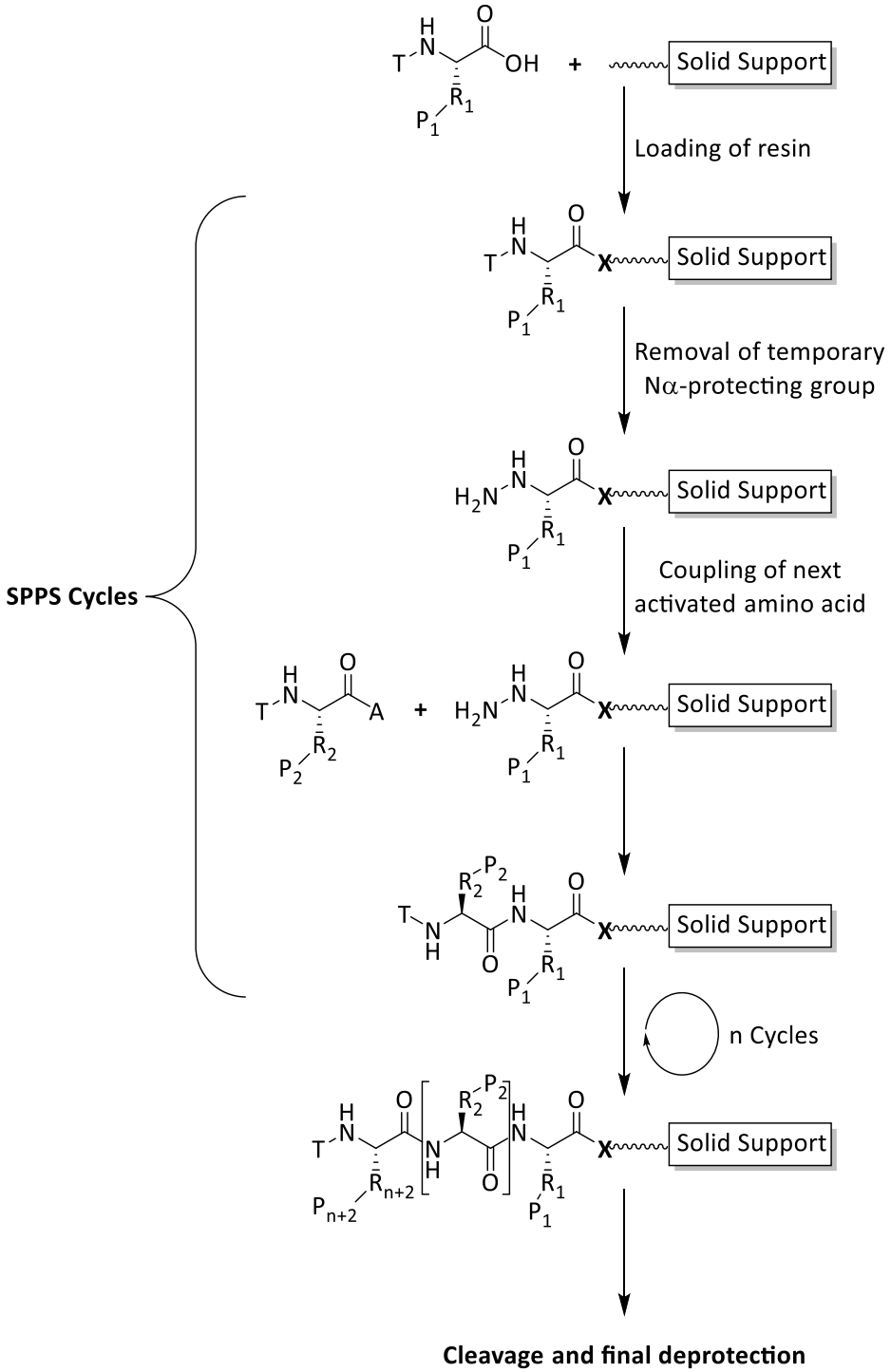


***Fig. 3**. Principle of SPPS. Abbreviations: P = permanent side chain protecting group, T = temporary Nα-protecting group, A = activating group, X = NH or O, ∿∿∿∿∿∿∿∿ = linker.*

sis by SPPS is sequence dependent. Peptides of more than 50 amino acids have been synthesized successfully but a peptide of fewer than 10 residues might be impossible to make [220]. Still, the applicability of SPPS is remarkable, with the availability of manual and automated systems for small and large-scale peptide synthesis of one or multiple peptides at the same time.

SPPS can be carried out in three different ways, i.e. sequential synthesis, convergent synthesis, and chemical ligation. In sequential synthesis, amino acids are added stepwise until the desired peptide is obtained. This strategy is most common for peptides of up to 50 amino acids in length. In chemical ligation, short unprotected peptides that are activated in such a way that they will react chemoselectively with only one group from the ligation partner, resulting in the site-directed formation of a new native peptide bond. Chemical ligation is employed mainly for the synthesis of large proteins and peptides [221–223].

2.2.3 Convergent peptide synthesis

In convergent synthesis (or hybrid approach), peptides of up to 50 residues are synthesized in a sequential manner and later ligated either by a suitable solid phase or solution phase coupling method [224]. This increases the chance of high peptide purity as the starting fragments are already purified and characterized and will also have properties very different from the desired product. Consequently, this is the preferred method for preparing large peptides [1,220]. The drawback is that the reaction rates of the coupling step are considerably lower than the coupling rates of the activated amino acids used in stepwise synthesis, primarily due to lower solubility of peptides containing protected amino acids. This has been solved to some extent by using a mixture of solvents. Moreover, as already mentioned in case of SPS, there is a risk of racemization at the coupling site during fragment ligation. The problem of racemization can be avoided by using proline or glycine at the *C*-terminus of the *N*-terminal fragment, which is not always possible. In that case, ligation is performed with Ala or Arg which are less likely to racemize during activation [1]. Both solution-phase and solid-phase variants are available for convergent peptide synthesis [2]. Fig. 4 illustrates a typical convergent peptide synthesis.

Chemical peptide synthesis is an active area of development. A great effort is going on to reduce the use of organic solvents and lessen the use of protecting groups [225–227]. Solvents are being developed that reduce racemization during activation and coupling [228]. Native chemical ligation (NCL), along with its variants, has been introduced to overcome the limitations of SPPS for the synthesis of large peptides and to reduce the risk of racemization [221,229].

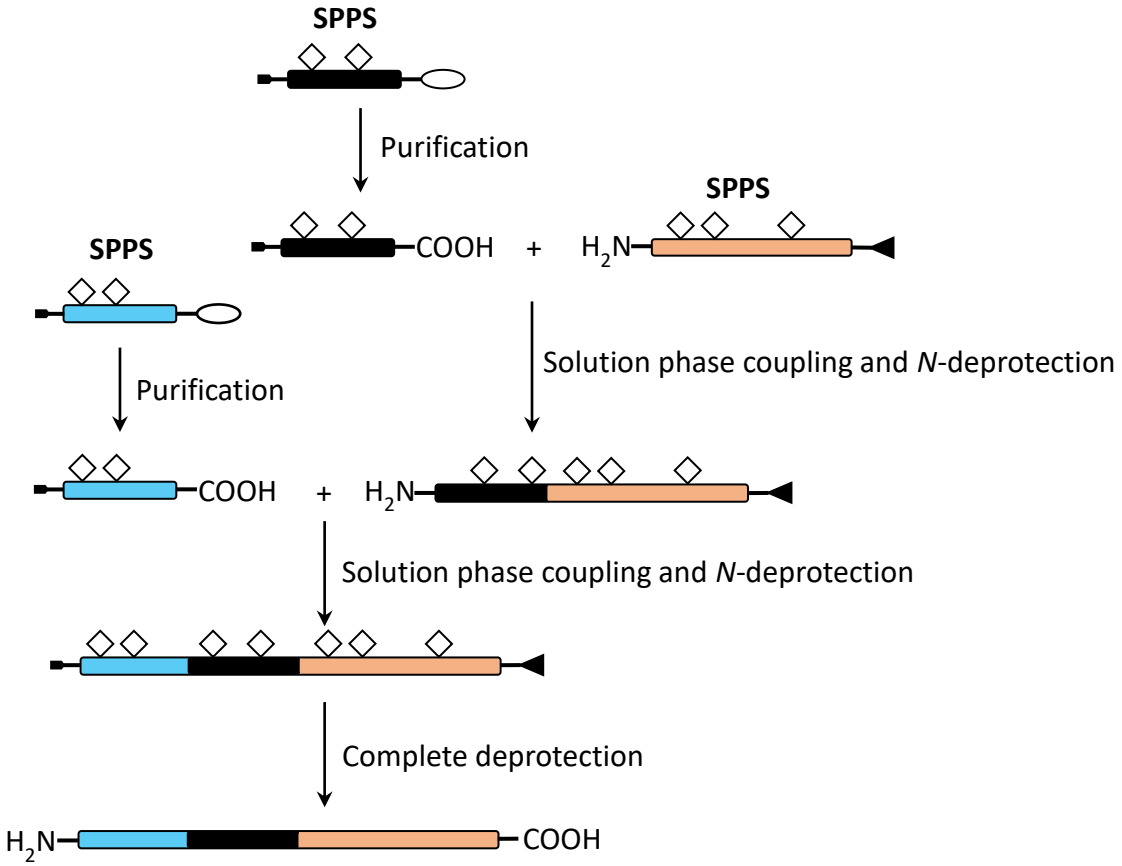


***Fig. 4**. Convergent approach for peptide synthesis using peptide coupling of fragments produced by SPPS. ▬ represents an N-terminal protecting group, ◀ represents a C-terminal protecting group, ◇ represents side chain protecting groups, ⬭ represents the solid support.*

2.3 Chemoenzymatic peptide synthesis

Although chemical peptide synthesis methods, especially SPPS, are mature strategies for making peptides, the problem of inevitable racemization during synthesis incurs heavy costs on the peptides because it leads to loss of expensive precursor fragments [1]. This demands alternative strategies and the answer is the formation of peptide bonds with the help of enzymes. Enzymes have a clear advantage over chemical synthesis in the sense that the racemization never happens, as the enzymes are regio- and stereo-selective at the coupling positions. This characteristic allows the use of amino acid building blocks with minimal or no protection, cutting the costs of reagents. Since enzymes catalyze coupling reactions under mild conditions, the use of toxic and hazardous chemicals in chemical synthesis is reduced, making the process environmental friendly [1,2].

An ideal option for enzymatic peptide synthesis would be the use of a universal C-N ligase having a high catalytic efficiency and that can couple all 21 proteinogenic

amino acids in every possible combination. This is not possible with enzymes obtained from nature [230]. In a living cell, ribosomal peptidyl transferase catalyzes the stepwise coupling of amino acids starting from the *N*-terminus of the growing peptide chain. The use of a ribozyme for *in vitro* peptide bond formation has been demonstrated [231]. However, the ribosomal peptidyl transferase is not a specific enzyme and catalyzes the coupling indiscreetly, irrespective of the amino acid side chain that is present. Nature controls the specificity with a multitude of factors including tRNA selectivity. Furthermore, the system requires coordinating factors that are involved in peptide chain elongation [232]. This explains the focus on enzymes that can selectively cleave a peptide bond – peptidases. Peptidases (or proteases) include an enormously wide range of enzymes that cleave peptide bonds by hydrolysis. In principle, we can fine-tune the reaction conditions to trigger these enzymes to catalyze the reverse reaction [233]. In fact, the synthetic capability of some proteases like pepsin, papain, and trypsin have since long been reported in the literature [234]. Additionally, proteases can catalyze esterification and transesterification reactions. This property makes them attractive catalysts for achieving very diverse conversions such as the kinetic resolution of racemic alcohols and carboxylic acids or the synthesis of glycoconjugates [235,236].

For the purpose of chemoenzymatic peptide synthesis, a peptidase should catalyze the coupling of smaller peptide fragments [233]. Mechanistically, one utilizes the principle of microscopic reversibility of the hydrolase mechanism for the coupling amino acids, dipeptides, and even larger peptides [235]. Typically, the enzymatic coupling comprises an acyl donor, which is either an amino acid or peptide that becomes the *N*-terminal part of the ligation product and an amine donor (or acyl acceptor) that becomes the *C*-terminal part [233,236]. In the case of protease catalyzed coupling reactions in aqueous media, the equilibrium of the reactions inclines toward the more stable hydrolysis products and synthesis yields are low. Fortunately, the conversion can be driven towards synthesis despite the unfavorable equilibrium by manipulating the reaction conditions, by product removal (e.g. precipitation), or by using an activated precursor fragment of which conversion to a coupled product is thermodynamically feasible [230,236]. Generally, as explained below, the synthesis can be approached in two different ways, i.e. thermodynamically controlled synthesis or kinetically controlled synthesis.

2.3.1 Thermodynamic and kinetic control of enzymatic peptide synthesis

In case of thermodynamic control (Fig. 5), the extent of the synthesis reaction (i.e. the reverse of hydrolysis) depends on the position of the thermodynamic equilibrium under the actual reaction conditions. The enzyme itself does not affect the equilibrium concentrations and the synthesis and hydrolysis reactions occur at the same time. Equilibrium can be shifted to synthesis by using excess of one of the reactants, by removing the products by precipitation, complexation, or extraction, by adding organic solvents to the reaction mixture that would change the pK_as of amino and carboxyl

groups, by using reverse micelles, or by employing anhydrous media with minimal water concentration. Also, the use of water mimics and optimization of the pH of the reaction medium may improve yields of coupling reactions [2,230]. One advantage of thermodynamically controlled synthesis is that proteases can be used irrespective of their mode of action. In spite of this advantage, thermodynamically controlled reactions require large amounts of enzyme and the reaction rates are usually low. Often, the use of organic solvents to shift the equilibrium further reduces the catalytic activity of the enzyme [237]. On the other hand, organic cosolvents also decrease the dielectric constant of the medium resulting in a lowering of the acidity of the carboxyl group and to some extent a reduction of the basicity of the amino group of the nucleophilic amino acid (or peptide). This increases the concentrations of non-charged species that take part in the coupling reaction [238].

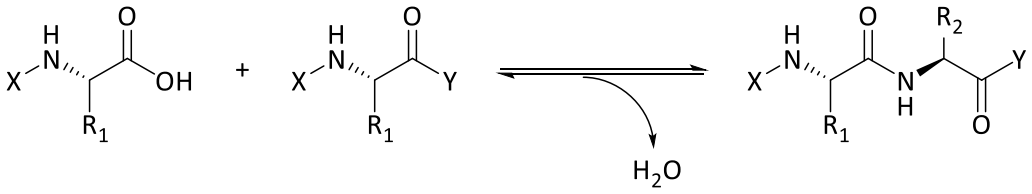


Fig. 5*. Thermodynamically controlled chemo-enzymatic peptide synthesis based on microscopic reversibility of the hydrolytic reaction.*

In kinetically controlled synthesis, an activated peptide is used that undergoes a peptidase-catalyzed coupling reaction with a second peptide at a much higher rate than hydrolysis, even in aqueous medium. Kinetically driven synthesis thus makes use of a high-energy *C*-terminally activated precursor peptide that is *N*-protected (Fig. 6). The catalyst usually is a (modified) serine or cysteine protease that is acylated by this *C*-terminally activated peptide fragment. For synthesis to proceed, the acyl-enzyme intermediate must preferentially (kinetically) be cleaved by the nucleophilic *N*-terminus of another peptide (or amino acid) as compared to cleavage by water. In that case, the peptidase-catalyzed reaction produces a coupled peptide, which may undergo (unwanted) slow secondary hydrolysis. Thus, in kinetically controlled synthesis, the concentration of synthetic product reaches a maximum until upon prolonged reaction times the formation of the hydrolysis product becomes significant. Ultimately, the system would slowly proceed to the thermodynamic equilibrium. Thus, the reaction should be stopped once the *C*-terminally activated precursor peptide acting as the acyl donor is consumed; after that hydrolysis becomes imminent. One can improve the transient synthetic yields by increasing the concentration of the nucleophile, by modifying the reaction conditions, or by optimizing the enzyme [235]. Still, the product must be removed at the right moment to avoid undesirable secondary hydrolysis.

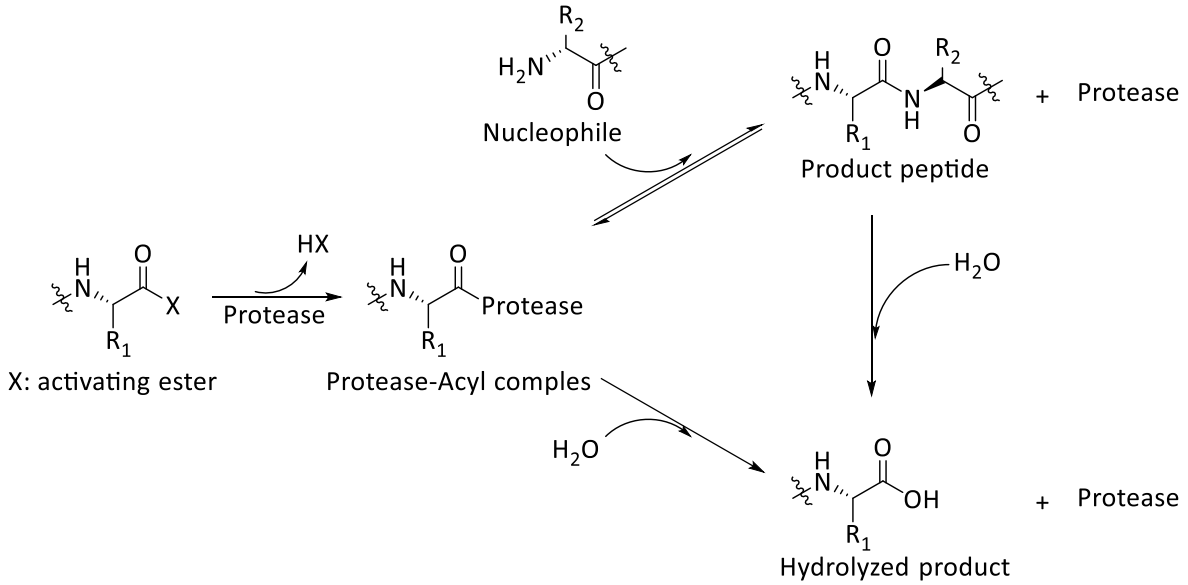


***Fig. 6**. Kinetically controlled peptide synthesis.*

For kinetically controlled peptide synthesis, proteases are used that form acyl-enzyme intermediates (Ac-E) when reacting with the *C*-terminally activated precursor [230]. For this purpose, serine and cysteine proteases are applied, such as subtilisin, trypsin, chymotrypsin, or papain. Other hydrolases than proteases, such as lipase and penicillin G acylase, can occasionally also be used for peptide synthesis. For synthesis to proceed, the formed acyl-enzyme intermediate must transfer its acyl group to the nucleophilic *N*-terminal amino group (N-H) of an incoming peptide. This amino group thus must outcompete water to allow peptide bond formation (Ac-N bond) instead of hydrolysis (Ac-OH). In other words, synthesis outcompetes hydrolysis of the acyl donor, even if the latter is thermodynamically preferred. Not surprisingly, the newly formed peptide (Ac-N) bond can slowly be hydrolyzed by the peptidase, again via formation of an acyl-enzyme intermediate. Thus, the ratio of synthesis by acyl transfer to the nucleophile compared to the rate of hydrolysis influences the product yield, and this S/H ratio should be as high as possible. Occasionally, peptidases can cleave the internal peptide bonds present in the oligopeptides that are used for the coupling reaction. Such side reactions occur due to the broad specificity of serine and cysteine proteases and can lead side products.

As illustrated in Fig. 7, kinetically controlled peptide coupling can happen at a high rate and give much higher yields as compared to thermodynamically controlled coupling. This is especially true in the initial phase of the reaction. As the maximum yield is reached, hydrolysis ensues and if the reaction is allowed to continue, the yield will decrease and the system will slowly approach the thermodynamic equilibrium.

Usually, the acyl donor (*N*-terminal fragment in the coupled product) is provided as a peptide of which the carboxylate group is activated in the form of an ester [236]. In the beginning of a conversion, this allows rapid formation of an acyl-enzyme and the yield of the synthetic product depends on the apparent ratio of the transferase: hydrolase rate constants. As conversion proceeds and product accumulates, the affinity of the enzyme for the activated acyl donor compared to the affinity for the coupled product becomes important, another reason why esters are attractive acyl donors. These enzyme-related

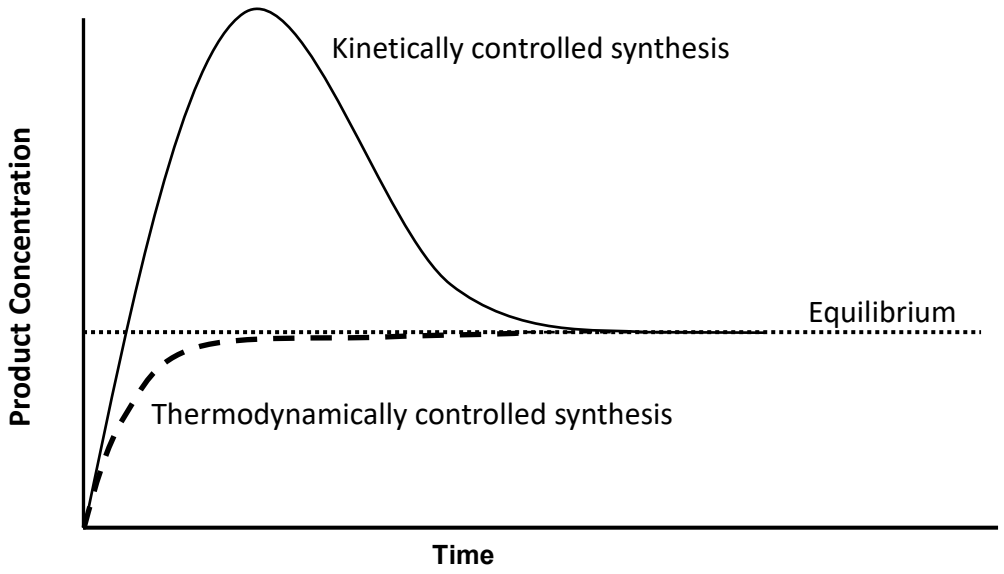


***Fig. 7**. Comparison of thermodynamically and kinetically controlled peptide synthesis.*

kinetic features explain the large differences between different enzymes with respect to synthetic yield that can be obtained. Still, with most enzymes, the reaction must be terminated as soon as the acyl donor is consumed since otherwise, hydrolysis of the product can commence.

Kinetically controlled synthesis is affected by pH and ionic strength. The nucleophile that attacks the acyl-enzyme complex (Fig. 6) must be uncharged, so a high pH is desirable. The optimum pH is usually close to the pK_a of the amino function of the nucleophile. The presence of organic solvents can help to dissolve the activated esters and also reduce the chances of hydrolysis of the ester or the peptide product. The specificity of the enzyme has a strong effect on the efficiency of kinetically controlled conversions and finding a good match between enzyme properties, peptide sequence at the coupling site, and nature of the activation group is of key importance.

As with chemical synthesis, chemoenzymatic peptide synthesis can be carried out by *N*- or by *C*-terminal extension [239]. In case of *N*-terminal extension, i.e. *C*→*N* enzymatic peptide synthesis, the *C*-terminus stays permanently protected and the amine of the growing peptide chain acts as the acyl acceptor (nucleophile). In this strategy, each amino

acid that is to be added must be *N*-protected to prevent self-condensation, and this *N*-terminal protecting group must be removed after each coupling step. This makes it a costly process because *N*-protection groups are expensive. In contrast, in case of *C*-terminal extension, i.e. *N*→*C* peptide synthesis, the *N*-terminus of the growing chain is permanently protected and at each step, an excess of a *C*-terminally protected (or non-activated) amino acid building block is used as the nucleophilic acyl acceptor. This strategy requires selective *C*-protection and deprotection (or activation) before the next coupling step. Synthesis of peptides with a protected or activated *C*-terminus can be achieved easily, for example via synthesis of carboxamides, which are cheap protection groups. The acyl group can be activated, for example in the form of alkyl esters, to increase the efficiency of nucleophilic attack by the amino group. Such a strategy would require additional, preferably enzymatic, procedures for modifying the *C*-terminus. Esterases, lipases, proteases, and amidases are good candidates for *C*-terminal esterification of amino acids [240–242]. In principle, both strategies require little to no side chain protection of amino acids owing to the selectivity of the proteases.

Part III. Peptide modification

3.1 Enzymatic peptide terminal modifications:

Peptide synthesis commonly utilizes protection and deprotection steps of the building blocks for controlled synthesis with avoidance of self-coupling reactions. Orthogonality of protecting groups is very important in order to protect and deprotect different functional groups independently of one another. In chemical synthetic routes, not only *C*-terminal and *N*-terminal protection but also side chain protection needs to be considered [243]. The use of enzymatic methods for protection, deprotection, and terminal modification has several advantages compared to chemical modification, owing to the possibility to use milder reaction conditions and by offering a selectivity that is superior in comparison to chemical methods [244]. In case of enzymatic reactions, side-chain protection is not required because of the enzyme's selectivity for the terminal functional groups of the main chain [1]. Several enzymatic methods for peptide or amino acid protection and deprotection reactions have been explored, as discussed below.

3.1.1 N^{α}-terminal modifications

N^{α}-amino groups can be blocked reversibly by acylation, alkylation, and alkyl-acylation and a large variety of amino-protecting groups has been described [245]. It is desirable that the protecting group should be removable under mild conditions to prevent the peptide from exposure to extremely acidic or basic conditions. A variety of alkoxycarbonyl-type (carbamates or urethane-type) *N*-protecting groups is available that are cleavable under different reaction conditions, of which benzyloxycarbonyl (Z or Cbz),

tert-butoxycarbonyl (Boc), and 9-fluorenylmethoxycarbonyl (Fmoc) are commonly used. Boc and Fmoc are often used for *N*-protection in SPPS in Boc/benzyl (Bn) and Fmoc/*tert*-butyl (^{t}Bu) strategies, respectively. The Z-group is often utilized in solution phase synthesis [211]. These groups are introduced by reacting the amino group with chloroformates or carbonic acid derivatives or mixed carbonate esters. Deprotection can be achieved by various methods e.g. hydrogenolysis in case of Z-group, protonolysis in case of Boc-group and β-elimination in case of Fmoc-group [245]. The groups are indeed employed under relatively mild conditions but there is a chance of dipeptide formation during the synthesis of *N*-protected amino acids due to the interference by the free α-carboxyl group. Hence, there is an ongoing search for protecting groups that can be cleaved off by enzymes. Such enzymatically cleavable protecting groups can be useful for the construction and manipulation of larger peptide units that need to be reacted under aqueous conditions, mainly for solubility reasons, but also in case of fragile peptide chains [2,244]. Moreover, an enzymatic procedure would be devoid of racemization and may be more cost-effective.

Initial attempts to introduce an enzyme-labile amino protecting group date back to 1955 when chymotrypsin was used for the removal of Bz-Phe (*N*-benzoylphenylalanine) from the tripeptide Bz-Phe-Leu-Leu-OH to obtain the H-Leu-Leu-OH dipeptide [246]. However, being an endopeptidase, the risk of peptide cleavage limits the applicability of chymotrypsin for this purpose. Later, trypsin, which has a narrower substrate specificity (i.e. only hydrolysis of Lys-X and Arg-X peptide bonds), was employed for the removal of Z-Arg as *N*-terminal protecting group. Z-Arg was coupled chemically with amino acid nitrophenyl esters to yield Z-Arg-aminoacylates (Z-Arg-X-ONp) that were further coupled chemically to peptide amides resulting in Z-Arg-protected peptides. At the last step of synthesis, trypsin was used to cleave the *N*-terminal Z-Arg yielding the final peptide product (Fig. 8) [247]. The method did not find general applicability owing to the difficult preparation of *p*-nitrophenyl esters. Nevertheless, it was utilized for the synthesis of oligo- and polypeptides via condensation of pre-formed peptide fragments, for instance in the preparation of β-lipotropin, oxypressin, Met-enkephalin and Glu-oxytocin [244].

The use of the above-mentioned proteases for *N*-terminal modification is accompanied by a risk of peptide bond cleavage at undesired internal sites in the peptide backbone. In principle, this risk can be overcome by using an enzyme that is not active as a protease in nature. Penicillin G acylase has been explored in this regard as the enzyme cleaves off phenylacetic acid (PhAc) from *N*-phenylacetyl peptides without hydrolyzing internal peptide bonds [248]. The enzyme has been used to synthesize phenylacetyl derivatives of amino acid esters and peptides, and for deprotection of the *N*-phenylacetyl peptides [244]. Immobilized penicillin acylase was used to selectively remove the phenylacetyl group from the *N*-phenylacetyl-aspartame to obtain aspartame

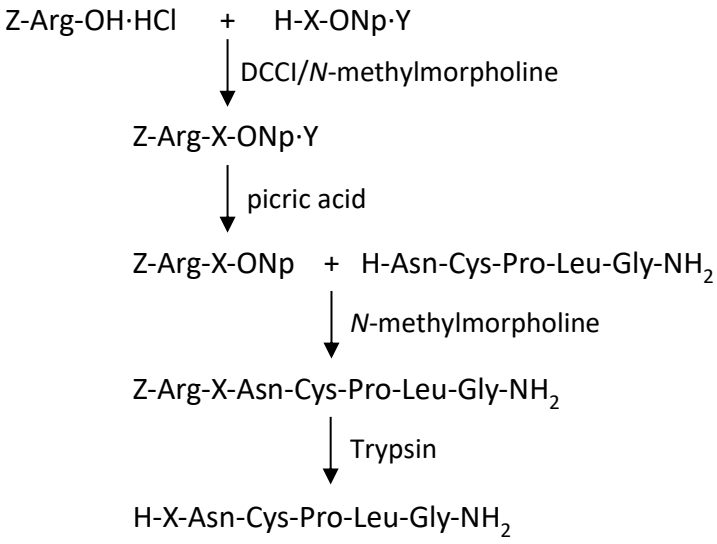


Fig. 8. *Scheme for synthesis of a hexapeptide utilizing Z-Arg as the N-terminal protecting group [247]. X = L-Glu, D-Glu, L-Ile or L-Tyr; Y = HCl, HBr or toluenesulfonic acid; DCCI = dicyclohexylcarbodiimide.*

as the final product [249]. Later, the enzyme was used for the formation of *N*-phenylacetyl protected amino acid esters, dipeptides, and tripeptides [250]. A fully enzymatic synthesis of leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu-*t*-butyl ester) has been described where penicillin acylase catalyzed both the *N*-PhAc-amino protection and deprotection steps (Fig. 9) [251]. Another enzyme, phthalyl amidase, was found to deprotect a variety of *N*-phthalimido substrates after the substrates were first partially hydrolyzed by weak base to their monoacids (Fig. 10) [252].

The benzyloxycarbonyl group (Cbz- or Z-group) has also been studied for enzymatic deprotection of *N*-terminally protected amino acids. The first example of a urethane hydrolyzing enzyme was reported in 1985 and it was named urethane hydrolase I [253]. However, the enzyme had a narrow substrate range and hydrolyzed only Z-Gly-OH efficiently. Moreover, a free carboxylate functional group was needed and a modification at the *C*-terminal end inhibited the enzyme activity. The same group of researchers identified similar enzymes, urethane hydrolases II, III and IV. All the enzymes exhibited narrow substrate specificity and accepted only Z-protected L-amino acids [254–257]. Of these four enzymes, urethane hydrolase IV exhibited the better substrate range, accepting Z-Leu, Z-Gly, Z-Ala, and Z-Ser as good substrates. In the same study, urethane hydrolase IV also deprotected Boc-protected amino acids, which is the only reported case of an enzymatic Boc deprotection [257]. Still, this proved to be inefficient for broader synthetic applicability [244].

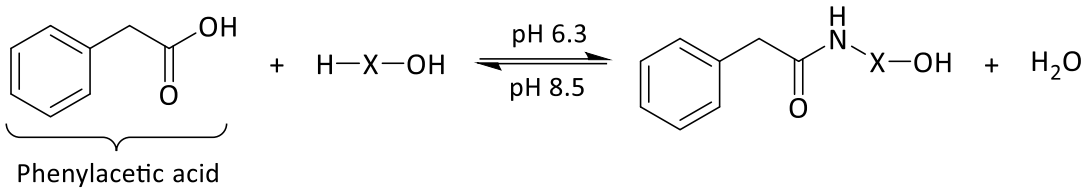


Fig. 9. *Penicillin acylase catalyzed synthesis of N-phenylacetyl amino acids or peptides. X = amino acid, dipeptide, or tripeptide* [249,250].

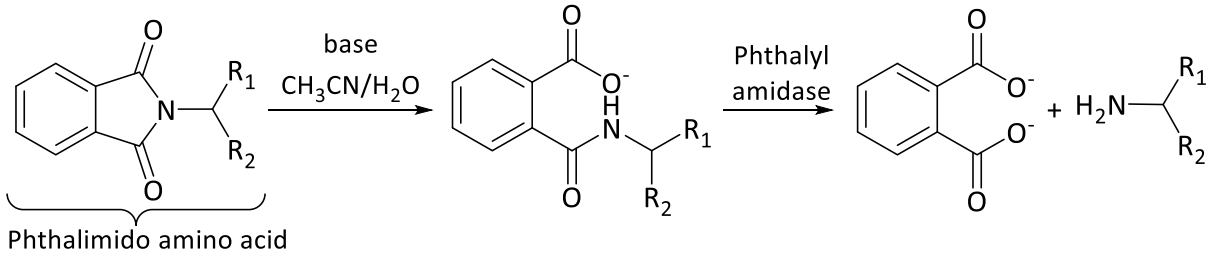


Fig. 10. *Phthalylamidase catalyzed removal of the N-phthalyl protecting group* [252].

Recently, urethane hydrolases have been reported that show broader substrate range. A urethane hydrolase from *Arthrobacter* species was found to remove the *N*-terminal Z-group from a variety of *N*-protected amino acids and related compounds. Like other urethane hydrolases, this enzyme required a free α-carboxylic acid group [258]. A urethane hydrolase from *Sphingomonas paucimobilis* has been reported that selectively cleaves Z-protected L-amino acids [259,260]. Another urethane hydrolase from *Burkholderia phenazinium* was reported by the same researchers to selectively hydrolyze Z-protected D-amino acids. Although both enzymes exhibit wider substrate range than previously reported for urethane hydrolases, the substrate range is still restricted to single amino acid substrates. Moreover, unlike previously reported urethane hydrolases, these enzymes show no requirement for a free α-carboxy terminal. While they have no demonstrated use in peptide synthesis, these enzymes are presumably applicable for enantiomeric resolution of racemic mixtures of Z-protected amino acids (Fig. 11) [261,262].

Penicillin G acylase, mentioned above to remove *N*-terminal phenylacetyl groups, can also catalyze deprotection of the Z-protected amino acids and peptides. Immobilized enzyme converted a variety of Z-protected amino acids and peptides including some dipeptides and tripeptides into corresponding unprotected peptides. Under the conditions used, conversion of the *C*-terminal methyl- or ethyl esters of *N*-terminally Z-protected substrates resulted in fully unprotected products owing to the saponification of ester moieties which took place before enzymatic *N*-deprotection under weak basic conditions [263]. This is disadvantageous as many peptide synthesis procedures use *C*-terminally esterified amino acids and peptides, which limits the applicability of penicillin acylase for *N*-deprotection of Z-groups. Other derivatives of the Z-group have also been developed and investigated for enzymatic deprotection. These include *p*-

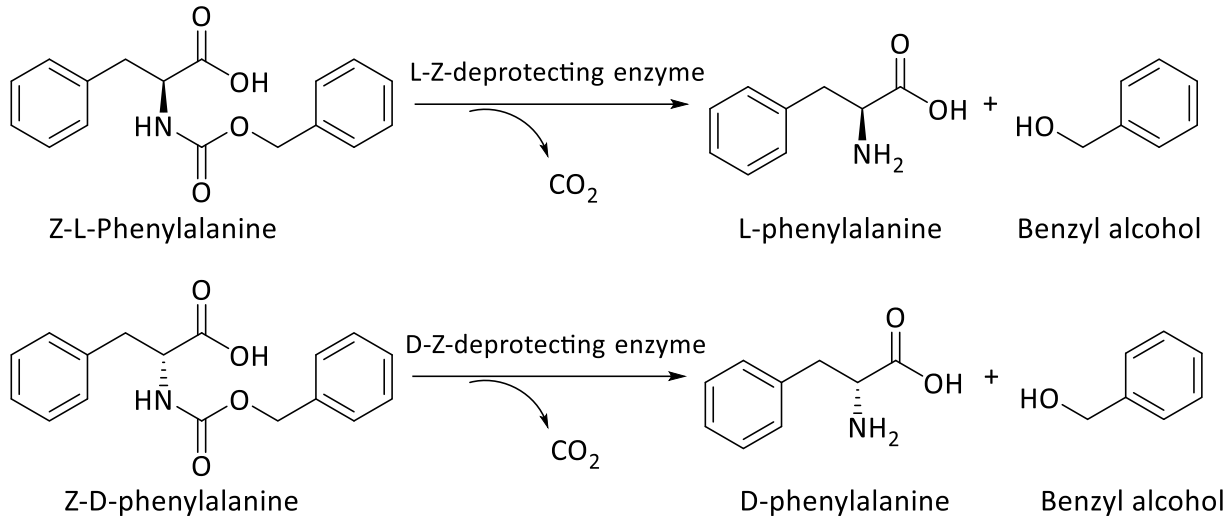


Fig. 11. *Z-deprotection of Z-D/L-phenylalanine by urethane hydrolases* [261]

acetoxybenzyloxycarbonyl (AcOZ) and *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) groups. The AcOZ- or PhAcOZ- protected amino acids can be obtained easily from *N,O*-bis(trimethylsilyl) amino acids and *p*-acetoxybenzyl chloroformate or *p*-phenylacetoxybenzyl chloroformate respectively [264]. Both AcOZ and PhAcOZ protecting groups include a spacer between the urethane group and the enzyme labile bond so that the enzyme labile bond is away from the target amino acid or peptide (Fig. 12). Cleavage of that bond causes the formation of a labile product that undergoes cleavage to yield the peptide with free *N*-terminus. This makes the urethane protecting group generally applicable as the target bond to be cleaved can be independent of the amino acid sequence and devoid of any steric hindrance due to amino acid or peptide structure. Moreover, unnatural amino acids could be used. [265–267]. For deprotection of AcOZ-peptides, acetyl esterase was used, but a lipase can also be applied for this purpose. For deprotection of PhAcOZ-peptides, penicillin G acylase was used due to its specificity for the phenylacetyl moiety. These protecting groups have been employed in the chemoenzymatic synthesis of *S*-palmitoylated and *S*-farnesylated *C*-terminus lipopeptides of the human *N*-Ras protein [265,268].

Other urethane derivatives like tetra-*O*-benzyl-D-glucopyranosyloxycarbonyl (BGloc), tetra-*O*-acetyl-D-glucopyranosyloxycarbonyl (AGloc), and tetra-*O*-acetyl-β-D-galactopyranosyloxycarbonyl (AGaloc) groups have been designed and studied for enzymatic removal. BGloc-protected amino acids are synthesized by reacting 2,3,4,6-tetrabenzylglucose with isocyanates (of respective amino acid allyl esters) followed by *C*-terminal allyl ester cleavage [269,270]. The removal of the *N*-protecting group occurs in two steps. First, the hydroxyl blocking group (the benzyl ether function) is removed by hydrogenation, followed by treatment with a glucosidase (in case of AGloc and BGloc) or a galactosidase (in case of AGaloc) (Fig. 13). Apart from enzyme lability under mild conditions, these protecting groups have the advantage of conferring solubility to the *N*-

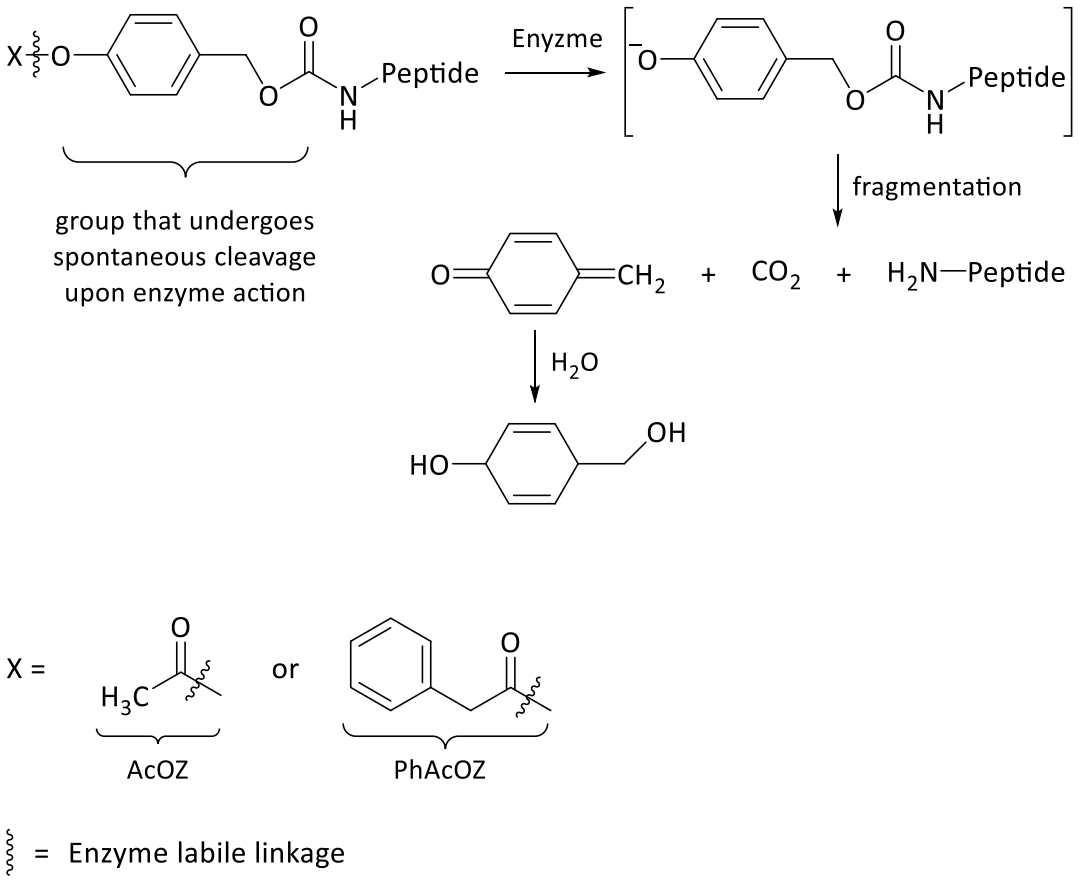


Fig. 12. *Enzymatic cleavage of spacer-based protecting groups AcOZ and PhAcOZ. Acetyl esterase (NaCl buffer, pH 7, 45°C) was used in case of AcOZ while PhAcOZ was cleaved by penicillin G acylase (phosphate buffer, pH 7.5, 25°C, $NaHSO_3$)* [244].

protected substrates. Moreover, these groups can be applied almost independently of the amino acid or peptides, in a manner similar to the spacer-containing urethanes, as the glycosidic C-O bond cleaved by the enzymes is not directly linked to the urethane [244].

The formyl group (For-) was studied for *N*-terminal protection because of its low cost, better solubility of the protected peptides in aqueous media, ease of introduction, and facile removal [271]. The *N*-formyl amino acids can be easily prepared chemically by reaction with formic acid in the presence of acetic anhydride and hydrolyzed by acid treatment (e.g. excess HCl in methanol) [272,273]. The latter treatment has the tendency to hydrolyze internal peptide bonds. Recently, peptide deformylases (PDFs) were used to remove the *N*-formyl group under mild conditions. During ribosomal protein synthesis, PDFs remove the formyl group from the first methionine of the nascent peptide chains [274]. The enzyme was examined in an improved strategy to produce aspartame from For-Asp and Phe-OMe monomers. Deprotection of For-α-Asp-Phe-OMe dipeptide (and its β-isomer) was performed by peptide deformylase (Fig. 14) [275]. In another example, an engineered PDF (EcPDF) was used in the synthesis of *N*-formyl-Tyr-Leu-Phe-NH_2. The strategy used thermolysin-catalyzed coupling of For-Leu with Phe-NH_2. The resultant dipeptide was deprotected at the *N*-terminus with EcPDF and used for subsequent

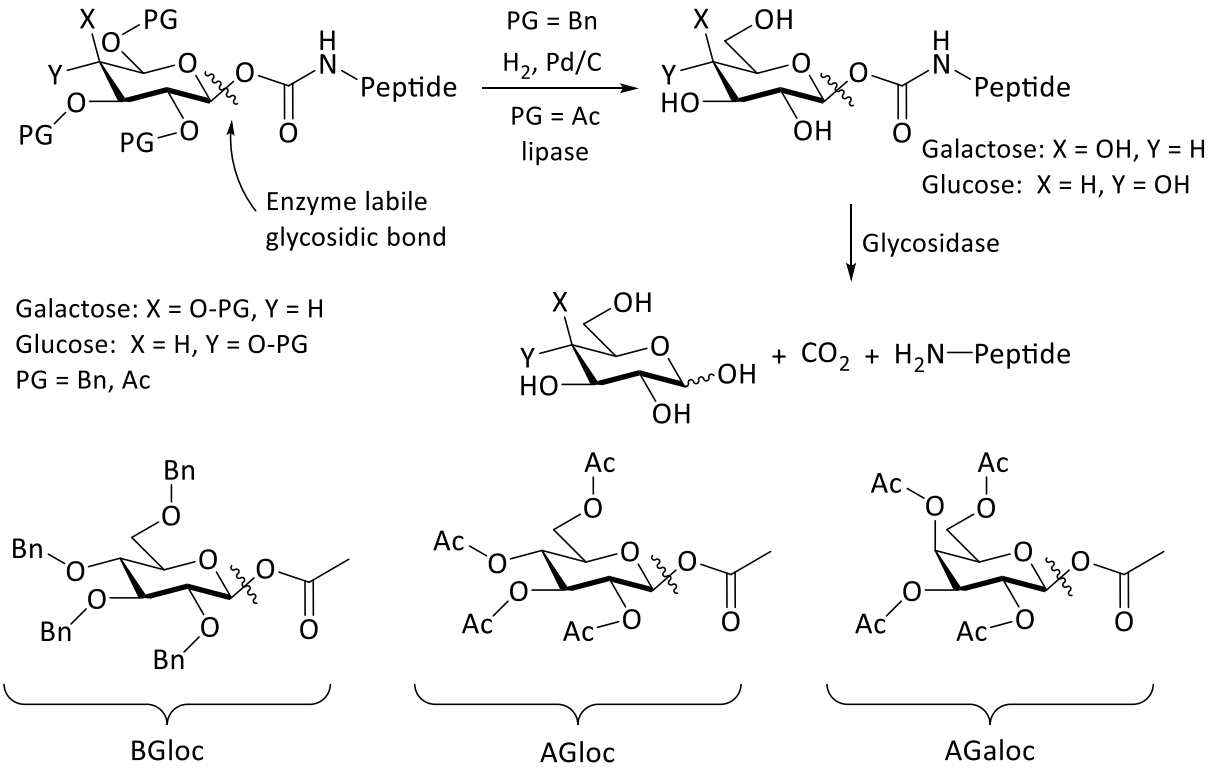


Fig. 13. *Two step enzymatic cleavage of carbohydrate based protecting groups BGloc, AGloc, and AGaloc* [244,269,270].

thermolysin-catalyzed coupling with For-Tyr, yielding the final tripeptide [276]. Studies show that the enzyme has a narrow substrate specificity and prefers formyl peptides with an *N*-terminal methionine. This limits the general applicability of peptide deformylase for chemoenzymatic peptide synthesis. Nevertheless, this does provide a platform for engineering PDFs and related enzymes with broader substrate specific and general applicability in peptide synthesis [277].

3.1.2 C^{α}-terminal modifications

In a usual SPPS synthesis, which occurs in the *C*→*N* direction, the first *N*-protected amino acid is anchored to a solid support resin via an ester or amide bond formed between the carboxyl group and the hydroxyl (or chloro) or amino function of the resin. This linkage is cleaved after the last step to remove the synthetic peptide from the resin. This implies that after each coupling step, the growing chain is subjected to *N*-deprotection such that the peptide is available for next coupling step (section 2.2.2, Fig. 2). Consequently, most research has been dedicated towards synthesis and removal of *N*-terminal protecting groups [2,243]. In a reverse *N*→*C* strategy either in solution phase synthesis or chemoenzymatic synthesis, a carboxyl protecting group would be required. The most common form of *C*-terminal modifications relevant to peptide synthesis is esterification [214]. A variety of protocols is available for esterification of amino acids at the carboxyl end. For example, methyl esters can be easily obtained by reacting amino acids

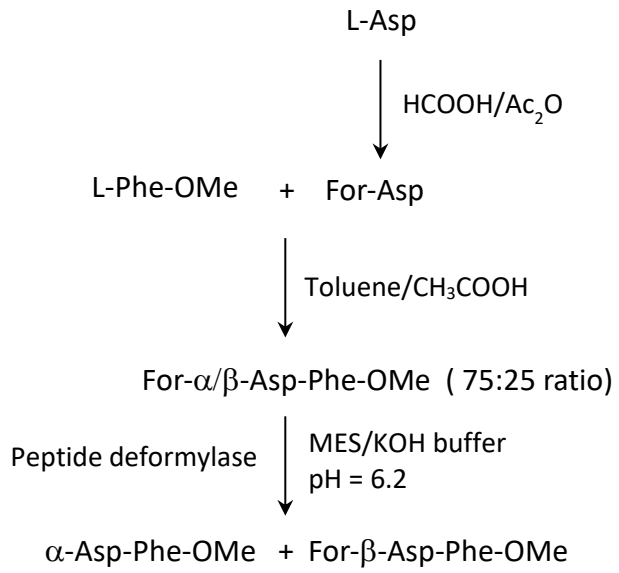


Fig. 14. *Aspartame synthesis using peptide deformylase for selective deprotection of formyl amino acids. MES = 2-(N-morpholino)-ethanesulphonic acid. The enzymatic deprotection resulted in a final yield of 44% for the α-isomer and <0.1% for the β-isomer* [275].

either with hot methanolic hydrochloric acid, or with methanol and thionyl chloride [214,240]. Methyl, ethyl, benzyl, *t*-butyl, phenyl, and phenacyl esters have been used in this regard and they can be cleaved by mild alkaline (methyl and ethyl) or acidic (*t*-butyl) hydrolysis or by hydrogenation (benzyl) [2,278].

The preference for an enzymatic route for *C*-terminal modification is based on the same arguments as stated in case of *N*-terminal modifications (section 3.1.1). Below we will discuss some important *C*-terminal enzymatic peptide modifications relevant to chemoenzymatic peptide synthesis.

3.1.2.1 Esterification

Esterase activity of chymotrypsin has been exploited to synthesize amino acid esters. Initially, chymotrypsin (immobilized on polyacrylamide or carboxymethyl cellulose) was used at low water concentration (20% water in alcohol) to synthesize *N*-acetyltyrosine ethyl ester. The synthetic yield, however, was low i.e. 40% [279]. To improve the yields, chymotrypsin was later employed in water: organic biphasic systems. Chymotrypsin was used in high ethanol concentrations to form ethyl esters of *N*-acetyltryptophan and *N*-acetyltyrosine in water: alcohol (single phase) and alcohol: chloroform (biphasic system), with higher yields reported for the biphasic system [280]. Chymotrypsin immobilized on porous glass was used to synthesize *N*-acetyltryptophan ethyl ester from *N*-acetyl tryptophan and ethyl alcohol in a water: chloroform biphasic system [281]. Chymotrypsin immobilized on silica gel was used to synthesize *N*-benzoylphenylalanine ethyl ester in biphasic systems using chloroform, benzene, carbon tetrachloride, or diethyl ether as organic solvents [282]. Silica-immobilized chymotrypsin

was also used to synthesize *N*-acetyltyrosine ethyl ester in a water: chloroform system [283]. In another example, agarose-immobilized chymotrypsin was used to convert *N*-acetyltryptophan to either the corresponding ethyl ester or to the phenylethyl ester by using 3-pentanone or 1,1,1,-trichloroethane as organic solvent, respectively [284]. Chymotrypsin immobilized on poly(vinyl alcohol) was used to convert *N*-acetyltyrosine to corresponding ethyl ester, in a hydrophilic organic solvent such as acetonitrile or ethanol. In these experiments, the esterase activity of chymotrypsin was reversed by either keeping extremely low amounts of water in the system or by continuously extracting the ester product into the organic phase. Free chymotrypsin was later used to esterify *N*-acetyltyrosine with a number of primary alcohols in different water miscible and immiscible organic solvents, with best conversation rates reported for the synthesis of *N*-acetyltyrosine methyl ester in acetone [285]. In another study, chymotrypsin immobilized on Chitopearl (porous chitosan beads) was utilized in a variety of organic solvents to synthesize *N*-acetyl-tyrosine ethyl esters. High esterification activities were reported with polar organic solvents (with the exception of methanol and DMF) while negligible esterification was observed in organic solvents such as chloroform and toluene [286]. Recently, free chymotrypsin was used to convert *N*-acetylphenylalanine to the corresponding ethyl ester in a hydrophilic ionic liquid medium (1-ethyl-3-methylimidazolium trifluoromethanesulfonate, [emim][Tf]) [287]. Although high conversion rates of up to 100% were reported in these studies, there is no wide application of chymotrypsin in esterification of amino acids or peptides within the context of peptide synthesis, probably due to the narrow substrate specificity of chymotrypsin, a property inherent to serine proteases.

Concurrent to the esterification studies with chymotrypsin mentioned above, papain was also studied for esterification because of its wider substrate range compared to chymotrypsin. Papain was used to convert a variety of *N*-terminally Boc-protected amino acids to the respective ethyl- and benzyl esters in a biphasic system [288]. Later, *N*-Boc-alanine was converted to a variety of esters using primary alcohols and diols in dichloromethane as the organic solvent. Allyl and cyanoethyl esters were also synthesized [289]. Synthetic yields were improved by immobilizing papain on XAD-57 and Sepharose for the synthesis of *N*-Boc-alanine and *N*-Boc-aspartic acid [290]. Immobilized papain was used in ethyl acetate as the organic solvent for the conversion of a variety of amino acids with different *N*-terminal protecting groups (Z, Boc, and methoxybenzyloxycarbonyl groups) to methyl, ethyl, benzyl, and butyl esters [291]. In another study, papain immobilized on Sephadex G-50 was used in 95% methanol to convert *N*-protected amino acids into the respective methyl esters [292]. Immobilized papain (on XAD-7 resin) also converted *N*-Boc-dipeptides to *N*-Boc-dipeptide ethyl esters in dichloromethane as organic solvent. It was observed that a hydrophobic amino acid at the penultimate position was a prerequisite to achieve esterification [293].

Formation of *N*-terminally Z-protected dipeptides was also catalyzed by papain (immobilized on Amberlite XAD-8) in ethanol containing 2% water [294]. Z-alanine-*sec*-phenethyl esters have also been synthesized by using Celite-immobilized papain from Z-alanine and 2-phenethyl alcohol, with and without organic solvents [295]. Amino acid glyceryl esters were also prepared by using papain. A variety of Boc- and Z- protected amino acids were converted to their respective glyceryl esters, which could be utilized in peptide coupling catalyzed by trypsin. Glyceryl esters of dipeptides were also synthesized in the same study, but with lower yields owing to secondary hydrolysis. Fig. 15 depicts a representative example of this synthesis [296].

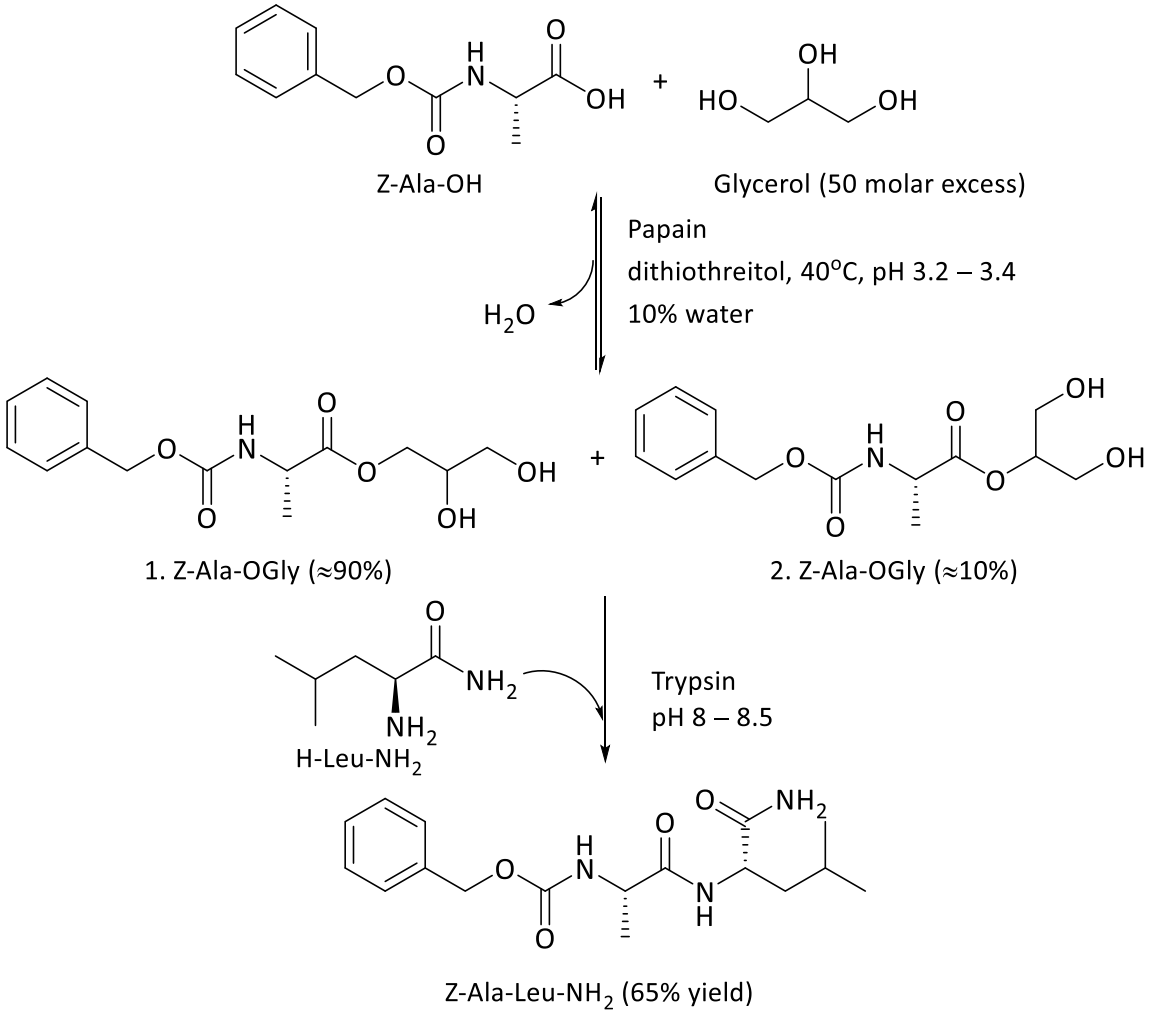


Fig. 15. *Formation of Z-Alanine glyceryl ester by papain and peptide coupling catalyzed by trypsin. Two structures of glyceryl esters are possible on esterification. Similar scheme was used to synthesize Z-Phe-OGl, Z-Ser-OGl, and Z-Thr-OGl esters and used for coupling via trypsin [296].*

Trypsin has also been studied to perform esterification, although not much data is available for direct esterification reactions catalyzed by trypsin as compared to its use in peptide synthesis. Trypsin was investigated to esterify *N*-acetyl-arginine with a variety of alcohols in different organic solvents. The highest yields obtained were for *N*-acetyl-

arginine ethyl ester in acetonitrile [297]. In another example, polyvinyl alcohol-immobilized trypsin was used to synthesize *N*-benzoyl-DL-arginine esters in hydrophilic organic solvents. Ethyl, 1-propyl, and 1-butyl esters were synthesized with highest yields reported for benzoyl-arginine ethyl ester [298].

Another protease examined for its use in esterification reactions of amino acids and peptides is subtilisin. In the same study where chymotrypsin was used for esterification of *N*- acetyl-tyrosine, subtilisin Carlsberg (also called as Subtilisin A or Alcalase) was tested for *C*-terminal esterification [279]. The enzyme was used in low water concentrations where chymotrypsin was ineffective. Maximum rates were observed in 5-15% (v/v) of water present in media containing ethanol:glycerol (1:1) as a cosolvent mixture, at pH 7.6 and room temperature (≈47% yield). In contrast to immobilized chymotrypsin, no conversion was observed when subtilisin was immobilized on different support media (carboxymethyl cellulose, aminoethyl cellulose, styrene-benzoate copolymer, ethylene-maleic anhydride copolymer). In a later study, subtilisin Carlsberg was used to convert *N*-acetyl-tyrosine in either ethanol, 1-propanol, 2-propanol, 1-butanol or 2-butanol to yield corresponding *C*-terminal esters. Maximum yields were reported for ethyl ester formation (90%) in presence of 2% (v/v) water in the reaction mixture [299]. Subtilisin BPN' was also used for esterification of *N*-acetyl-tyrosine. Unlike subtilisin Carlsberg, this enzyme was inactive in ethanol but activity was found when it was coupled to polyvinyl alcohol and reactions were done in the presence of added polysaccharides such as chitin or chitosan [299].

Celite-immobilized Alcalase was used to synthesize Z-Tyr-*sec*-butyl ester, Z-Tyr-*sec*-phenethyl ester, and Z-Ala-*sec*-phenethyl ester from their corresponding Z-amino acids and pure 2-butanol or 2-phenethyl alcohol used as organic solvent as well as the reactant [295]. Free subtilisin was used to synthesize esters of a variety of *N*-terminally Boc-protected amino acids and unprotected peptides after solubilizing in high amounts of 1,3-propanediol or 1,4-butanediol at low water content. High yields of diol-mono esters were reported in this case. The amino acid esters were further used in enzymatic fragment coupling by different proteases (Fig. 16) [300]. Recently, Nuijens *et al*. investigated Alcalase-CLEA to catalyze *tert*-butyl ester formation in neat *tert*-butyl alcohol as the solvent. High yields of *N*-protected-peptide-*tert*-butyl esters were obtained without any hydrolysis of the peptide when water was continuously removed from the reaction mixture. Most of the peptides were well accepted as substrates but amino acids with a tertiary β-carbon (e.g. threonine, isoleucine, and valine) were not accepted as good substrates (Fig. 17) [301].

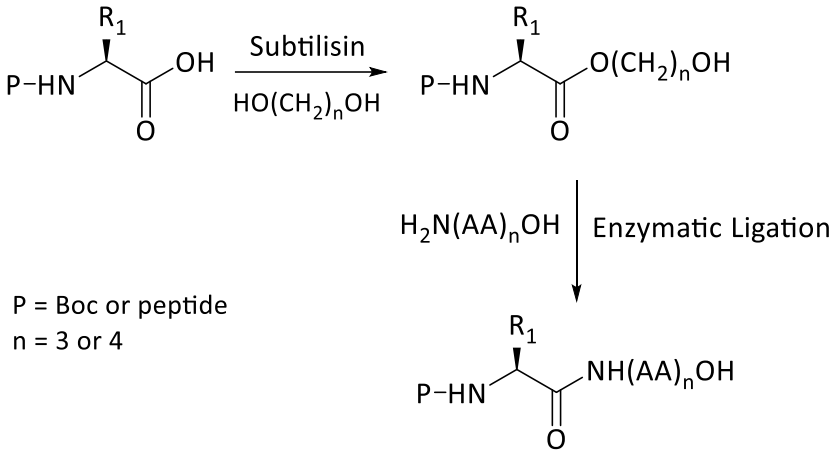


Fig. 16. *Subtilisin catalyzed esterification of Boc-protected peptides and unprotected peptides and their subsequent use in enzymatic fragment ligation [300].*

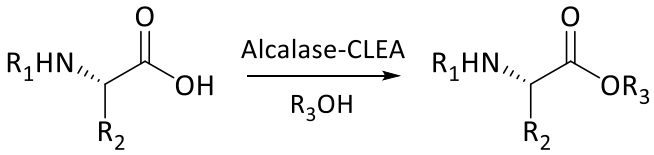


Fig. 17. *Alcalase-CLEA catalyzed esterification of N-terminally Z-amino acids to respective t-butyl, methyl, benzyl esters. The protocol was also used to synthesize allyl and 2-(trimethylsilyl)-ethyl esters of Z-, Boc-, or Fmoc-protected aspartic acid and glutamic acid [301].*

3.1.2.2 Transesterification

Transesterification was investigated initially in a comparative study of subtilisin Carlsberg, subtilisin BPN' and α-chymotrypsin, for esterification of *N*-acetyl-tyrosine. Both subtilisin Carlsberg and immobilized subtilisin BPN' were found to be superior compared to α-chymotrypsin for the synthesis of *N*-acetyl-tyrosine ethyl ester in ethanol containing 4.8% water, at 30°C (83% yield)[299]. In an extension of the same study, subtilisin Carlsberg, subtilisin BPN' and chymotrypsin were immobilized on Chitopearl (porous chitosan beads). The immobilized enzymes exhibited better reaction rates than free enzymes for transesterification of *N*-acetyl-tyrosine-OMe to Na-acetyl-tyrosine-OEt using ethanol with a water content as low as 2.9% in the reaction mixture [286]. α-Chymotrypsin was also used to synthesize tyrosine glyceryl esters by transesterification from tyrosine-methyl ester using 60% (v/v) glycerol in water as reaction medium, at pH 7 and 40°C, with a high yield (80%) [302].

Transesterification using α-chymotrypsin has been investigated in ionic liquid media, primarily to minimize the destructive effects of water-miscible organic solvents such as alcohols [303]. An example is the synthesis of *N*-acetyl-tyrosine propyl ester in ionic liquid media. Transformation occurred in a kinetically controlled process in which the

nucleophile competes with water to achieve esterification instead of hydrolysis. Ester synthesis was optimized by minimizing water content and by addition of triethylamine. A variety of ionic media were tested, of which [Bmim$^+$][BF_4^-] gave best yields (Fig. 18) [304]. Recently, both subtilisin and α-chymotrypsin, immobilized on chitosan, were used to synthesize *N*-acetyl-phenylalanine-propyl esters from the corresponding ethyl esters. Transesterification was performed in ether-functionalized hydrophobic ionic liquid media ([$Me(OEt)_3$-Et_3N][Tf_2N], [$Me(OEt)_3$-Et-Im][Tf_2N], and [$Me(OEt)_3$-Me-Et-Im][Tf_2N]) containing 1-propanol, 10-15% water, at 50°C [305]. Ionic liquids are gaining interest in chemical synthesis procedures as well as enzymatic synthesis due to their nonvolatile nature, thermal stability, and low environmental impact. However, future work is required to make a fully enzymatic synthesis feasible in pure ionic liquid media.

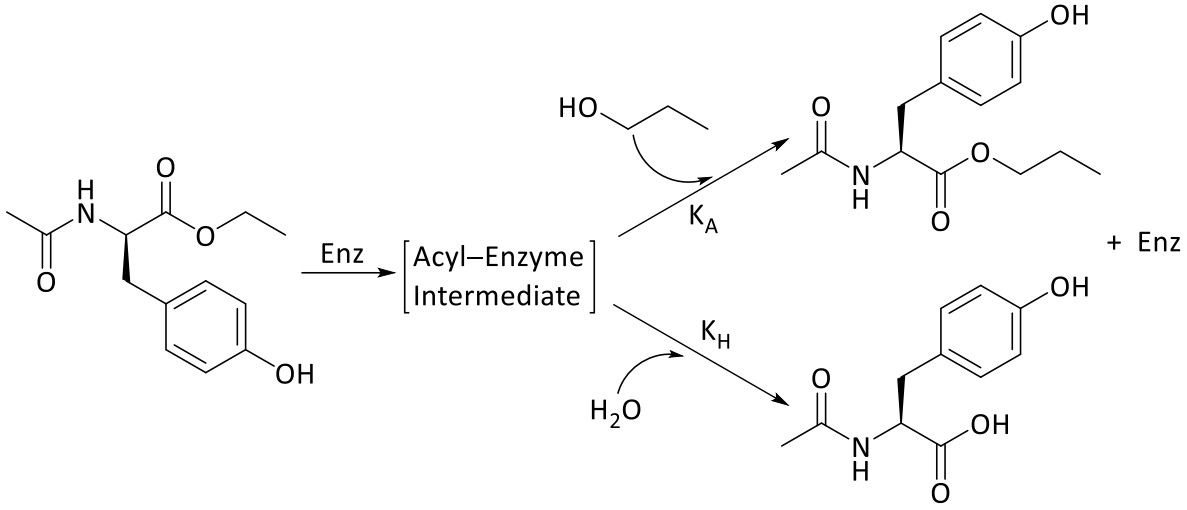


Fig. 18. *Transesterification of N-acetyl-tyrosine ethyl ester to N-acetyl-tyrosine-propyl ester. Transesterification occurred in different ionic liquids: [Emim$^+$][BF4$^-$], [Emim$^+$][Tf_2N^-], [Bmim$^+$][BF_4^-], [BMIm$^+$][PF_6^-], [MTOA$^+$][TF_2N^-]. Reactions were performed at 50°C, with 2% water (v/v) in the medium.*

Transesterification was accidentally observed during peptide synthesis catalyzed by Alcalase-CLEA by Nuijens *et al*. During enzymatic coupling of Z-Phe-OMe and Leu-O*t*-Bu in *tert*-butanol as solvent, Z-Phe-O*t*-Bu was obtained as a byproduct [301]. The discovery was interesting as chemical synthesis of *tert*-butyl esters is rather laborious and requires harsh conditions [306]. Consequently, transesterification was investigated for the conversion of various peptide *tert*-butyl esters (obtained via SPPS) to methyl, ethyl, or benzyl esters in anhydrous media (Fig. 19). The esters served as acyl donors for the subsequent enzymatic coupling step. The strategy was successfully used to synthesize anti-inflammatory peptides (For-Met-Leu-Phe-OMe and Boc-Phe-Leu-Phe-Leu-Phe-OH). The multistep synthesis scheme included Alcalase-CLEA catalyzed formation of methyl ester, initial coupling reactions, transesterification of *tert*-butyl esters to methyl esters, a second coupling, and hydrolysis to the final product. Similarly, the thermolysin

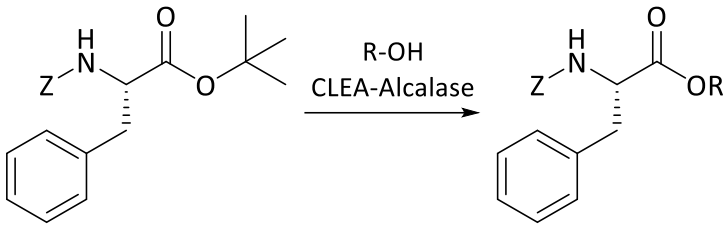


Fig. 19. *Transesterification of Z-Phe-O-tBu catalyzed by Alcalase-CLEA into primary alkyl esters. The procedure was also successfully applied to convert dipeptides, for example, Z-Phe-Leu-O-tBu, Z-Ala-Leu-O-tBu, Z-Ala-Phe-O-tBu, and Z-Gly-Phe-O-tBu, to activated esters [242].*

tripeptide assay product, Cbz-Phe-Leu-Ala-OH, was synthesized by initial coupling and later hydrolysis of the peptide to obtain the product [242].

In order to facilitate peptide synthesis with sterically demanding and non-proteinogenic acyl donors, and poor nucleophiles, carboxyamidomethyl (Cam) and trifluoroethyl (Tfe) esters were synthesized using the same Alcalase-CLEA. Cam and Tfe ester syntheses were also catalyzed by CalB lipase. High yields are reported for both enzymes, and esterification and peptide bond formation could be catalyzed in the same reaction mixture by using CalB and Alcalase-CLEA simultaneously (Fig. 20) [241].

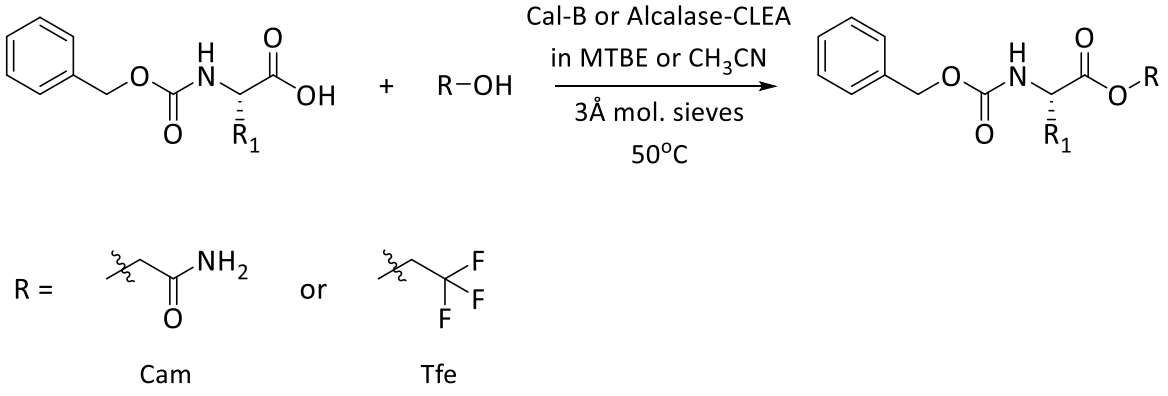


Fig. 20. *Cam and Tfe ester syntheses catalyzed by CalB (lipase from Candida antarctica) or Alcalase-CLEA [241].*

Another *C*-terminal modification is the formation of peptide thioesters. Peptide- and amino acid thioesters are important building blocks for native chemical ligation in peptide synthesis and macrolactamization of peptides [307,308]. Peptide thioesters can be synthesized chemically, for example by solid phase synthesis, but the subsequent removal of protecting groups requires harsh conditions [309]. Enzymatic synthesis of peptide esters is favorable as reactions would be possible under mild conditions. Subtiligase (a subtilisin BPN' variant with the Ser221Cys and Pro225Ala mutations which

improve synthetic performance) has been used for transesterification of peptide glycolate esters to peptide thioesters in the presence of *N*-acetylcysteamine as thiol [310]. Quaedflieg *et al*. used subtilisin in a neat organic solvent for the transesterification of *N*-terminally protected peptide esters by a variety of thiols (e.g. benzyl mercaptan or 3-mercaptopropionic acid ethylester) to obtain the corresponding peptide thioesters [311].

3.1.2.3 De-esterification

Initial attempts to enzymatically deprotect carboxyl groups focused on the use of chymotrypsin, trypsin, and thermolysin. The esterase activity of chymotrypsin and trypsin have been investigated for hydrolysis of several peptide methyl, ethyl, and *tert*-butyl esters. The investigations reported successful transformations not only with peptides having enzyme specific amino acids at the *C*-terminus but also with different ones. However, cleavage of internal peptide bonds was imminent in cases where trypsin- or chymotrypsin-labile sequences were present in the peptide. Moreover, many peptides were poor substrates or were not accepted at all [244,312,313]. Thermolysin was explored to selectively cleave Leu-Gly-Gly-OEt from undecapeptide ester (Boc-Gln-Thr-Lys-His-Pro-Lys-Lys-Gly-Leu-Gly-Gly-OEt). The tripeptide served as a supporting peptide in the synthesis strategy and the resulting octapeptide consisted of hydrophilic residues [314]. The idea did not find general applicability due to the broad substrate range of thermolysin that poses a huge risk of internal peptide cleavage. Moreover, the enzyme was restricted to the use of the specific tripeptide ester (Leu-Gly-Gly-OEt) for enzymatic deprotection [244,314].

The problem of endopeptidase activity of enzymes could be reduced by using carboxypeptidase Y (CPY). The enzyme has both peptidase and esterase activities but at different optimum pHs. Carboxypeptidase Y (immobilized on CL-Sepharose or concanavalin) was first used in stepwise synthesis of a tripeptide Leu-Phe-Phe-OEt. At each step before coupling, the *C*-terminal ethyl ester was removed by CPY at pH 8.5 and subsequently coupled with the next amino acid ethyl ester [316]. In another example, immobilized CPY was used to remove the ethyl ester group from the starting peptide, *N*-terminally blocked by carboxymethyl poly(ethylene glycol) (CM-PEG), CM-PEG-Gly-Met-OEt. The resulting peptide was stepwise elongated at the *C*-terminus by CPY-catalyzed removal of the ethyl ester group before each coupling step. The final peptide was released by cyanogen bromide cleavage (Fig. 21) [315]. Another prospect is the use of enzymes that have high esterase/protease ratio. Examples of such enzymes include alkaline protease from *Bacillus sublitilis* DY (similar to subtilisin Carlsberg), Alcalase (subtilisin A, a serine endopeptidase from *Bacillus licheniformis*), and thermitase (a serine protease from *Thermoactinomyces vulgaris*). These enzymes have high esterase/protease ratios and accept a variety of peptide esters as substrates [317–320].

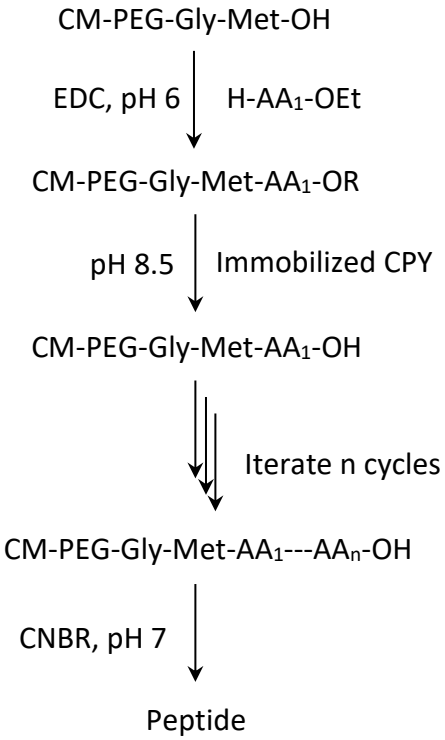
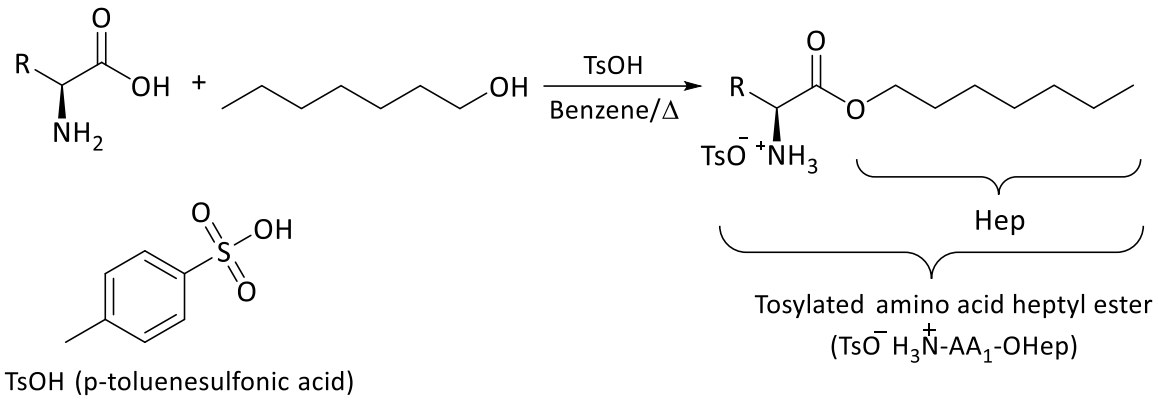


Fig. 21. *Peptide de-esterification by Sepharose-immobilized carboxypeptidase Y (CPY) as a step in chemoenzymatic peptide synthesis. ECD = 1-ethyl-3(3-ethyldimethylaminopropyl) carbodiimide [315].*

The risk of protease dependent peptide hydrolysis can be avoided by using enzymes lacking amidase activity, e.g. lipases. Lipases incur several advantages over proteases. Many commercially available and inexpensive lipases accept a wide range of substrates including D-amino acids, and work at the interface of water and organic solvents [322]. An example of a lipase-cleavable *C*-terminal protecting group is the heptyl (Hep) ester. Heptyl esters were synthesized by azeotropic esterification of amino acids with n-heptanol in p-toluenesulfonic acid. The resulting crystalline salts were coupled with *N*-terminally Z-, Boc-, and Aloc- protected amino acids to give the respective dipeptide heptyl esters (Fig. 22). Lipase from the fungus *Rhizopus niveus* hydrolyzed *C*-terminal heptyl esters from the resulting dipeptides without interfering with the *N*-protecting groups or internal amide bonds (Fig. 22). However, the conversion was low if there was a hydrophobic *C*-terminal amino acid in the dipeptide (e.g. phenylalanine) [321]. Another lipase from *Mucor javanicus* was used to deprotect heptyl esters present in labile amino acid derivatives and the reaction proceeded without any undesirable side reactions [323]. This lipase was used for chemoenzymatic synthesis of *O*-glycopeptides which are very sensitive to acid- or base- treatments during chemical deprotection steps [323,324]. It was observed that the activity of lipase was lower in cases where the hydrophobicity of the peptide was higher (making substrates less soluble and accessible to the lipase), a problem partly due to the high hydrophobicity of the heptyl group.



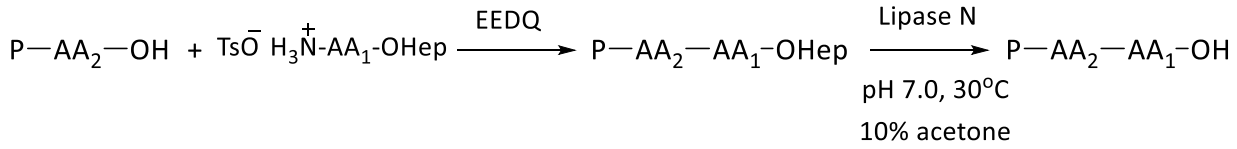


Fig. 22. *Chemical synthesis of amino acid heptyl ester and subsequent coupling to yield C-terminal peptide-heptyl esters. Lipase N (from Rhizopus niveus) catalyzed the removal of heptyl ester to yield peptides with free carboxyl end [321]. AA = amino acid. P = Z-, Boc- or Aloc- protecting group. R = H (Gly), CH_3 (Ala), $CH(CH_3)_2$ (Val), $CH_2CH(CH_3)_2$ (Leu), $CH(CH_3)CH_2CH_3$ (Ile), CH_2OH (Ser), $CH(OH)CH_3$ (Thr), $CH_2C_6H_5$ (Phe). EEDQ = ethyl-2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (condensing agent).*

Furthermore, hydrophobic *N*-protecting groups like Fmoc also added to the hydrophobicity of peptides [321,324].

To make the substrates more accessible to lipases and increase the solubility of peptides in general, hydrophilic heptyl analogs were used as carboxyl protecting groups, for example by using a 2-*N*-(morpholino)ethyl ester (MoEt ester) or a (methoxyethoxy)ethyl ester (MEE ester). Another lipase (lipase CE from *Humicola lanuginosa*) was found to hydrolyze peptide-MEE esters when proline was the *C*-terminal amino acid, which was not possible with lipase N (Fig. 23) [325]. This prompted the use of lipase-labile polyethyleneglycol (PEG) esters. Fmoc-peptide-PEG esters were hydrolyzed at the *C*-terminus to release the Fmoc-peptides in neutral media [325]. Lipases and lipase-labile *C*-terminal protecting groups have been used to synthesize small peptides and peptide conjugates including glycol- and nucleopeptides [326,327]. A lipase from *Candida antarctica* (Cal-A) was used for the removal of methyl- and benzyl- esters of *N*-(Boc/Z)protected amino acids and peptides, requiring longer reaction times and giving lower yields as compared to amino acid substrates [328].

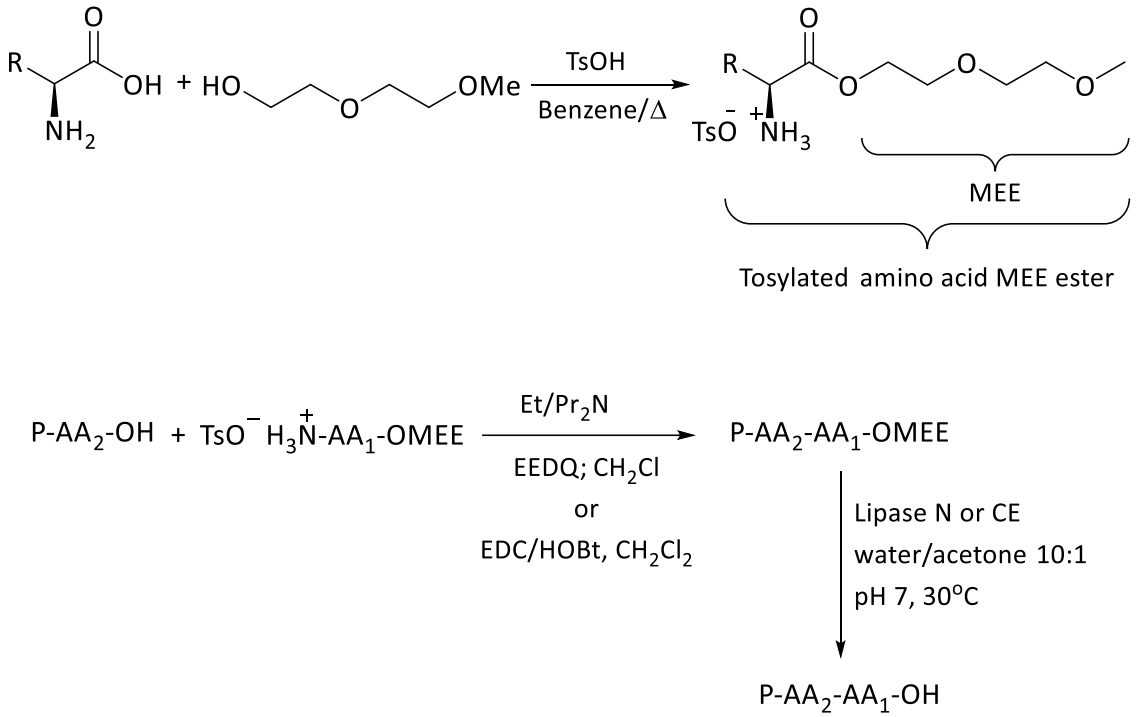


Fig. 23. *Chemical synthesis of amino acid MEE esters and subsequent coupling to yield C-terminal peptide-MEE esters. Lipase N (from Rhizopus niveus) catalyzed the removal of the MEE ester group to yield peptides with a free carboxyl end. AA = amino acid. P = Fmoc-, Z-, or Aloc- protecting group. TsOH = p-toluenesulfonic acid. R = CH_2OH (Ser), $CH(OH)CH_3$ (Thr). EEDQ = ethyl-2-ethoxy-1,2-dihydro-1-quinolinecarboxylate (condensing agent). EDC = 1-ethyl-3-(dimethylaminopropyl)carbodiimide. HOBt = 1-hydroxybenzotriazole.*

In addition to lipases, butyrylcholine esterase was also used to remove the *C*-terminally protected peptide choline esters groups. Choline esters were synthesized to enhance the solubility of hydrophobic peptides (another hydrophilic analog of the heptyl ester protecting group). Peptide choline esters were synthesized either by reacting peptide 2-bromoethyl esters with triethylamine or by condensing amino acid choline esters with amino acids having a free carboxy end. As with lipases, ester removal occurred under mild conditions without affecting any internal amide linkages [329]. Choline esterases such as butyryl choline esterase (BChE) and acetylcholine esterase (AChE) have been used to hydrolyze sensitive peptide conjugates, including phosphorylated and glycosylated peptides, lipidated peptides and nucleopeptides [327,329,330]. An esterase from *Bacillus subtilis* (BsubpNBE) was shown to selectively hydrolyze *C*-terminal *tert*-butyl esters of *N*-(Boc)-amino acids, but the enzyme did not accept any peptide *tert*-butyl ester as substrate [331]. Another esterase from *B. subtilis* (BS2) was found to hydrolyze *C*-terminal methyl, benzyl, and allyl esters of *N*-(Boc/Z) protected amino acids and dipeptides with high product yields [328].

3.1.2.4 Amidation

Carboxamides are also considered as suitable carboxyl protecting groups for peptides and amino acids. *C*-terminal amides are abundantly found in natural peptides where the amide group serves to protect the peptide from exoprotease activities and makes the peptide more biostable. Moreover, many therapeutic peptides and hormones are active only in the form of *C*-terminal carboxamides [2,332,333]. These characteristics have triggered research on the introduction of carboxamide groups in peptide modification reactions, which is also relevant in case of chemoenzymatic peptide synthesis in the *N*→*C* direction. In the *N*→*C* directed peptide synthesis, amino acid building blocks are used that are temporarily protected at the *C*-terminus and are usually activated as alkyl esters before enzymatic coupling by a protease [334].

Carboxypeptidase CPD-Y has been used for peptide *C*-terminal amidation by accepting amino acid amides as the nucleophiles, thus extending the non-activated peptide with an amino acid amide. CPD-Y has very low peptidase activity at high pH (and still a high esterase activity). Catalysis proceeds via an acyl-enzyme intermediate. This property was exploited by using different nucleophiles at high pH (e.g. ammonia or an amino acid amide) to produce amino acid amides with up to 100% yield (Fig. 24) [335]. Transacylations with *N*-benzyl-Ala-OMe and *N*-benzyl-Ala-OEt esters with different amino components was also investigated. CPY-Y catalyzed the conversion of such methyl and ethyl esters into amides, giving the highest yields with amino acid amides (70-98%) as the nucleophiles. Among the amino acid amides tested as amine components, only isoglutamine amide and prolineamide were not accepted for amide bond formation. Furthermore, none of the dipeptides included in the study were accepted as nucleophiles, in agreement with the exoprotease activity of the enzyme [336]. The transpeptidation activity of CPD-Y was used to modify growth hormone releasing factor peptide GRF(1-29)-OH to the amidated peptide GRF(1-29)-NH_2 using a *C*-terminal

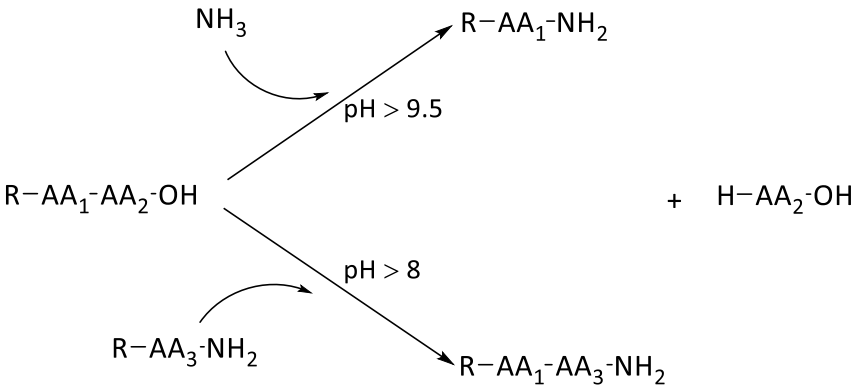


Fig. 24. *Summary of transacylation reactions catalyzed by carboxypeptidase Y with different nucleophiles. Best yields were reported for Z-Ala-Ala-OH as the substrate while glycine amide (AA_3) was the nucleophile (100% yield for Z-Ala-Gly-NH_2). AA_2, in this case Ala, serves as the leaving group [335].*

alanine in peptide GRF(1-28)-A-OH as the leaving group and Arg-NH_2 as the nucleophile [337].

Industrial alkaline protease (Alcalase) has been used to catalyze the synthesis of amino acid- and peptide amides from the respective *N*-protected amino acid- and peptide methyl esters. Alcalase catalyzed this conversion with ammonium chloride in triethylamine as a source of nucleophilic ammonia in anhydrous *tert*-butanol. Amide formation occurred in a kinetically controlled manner where the nucleophile ammonia attacked the acyl-enzyme intermediate. Synthetic yields were substrate dependent (44-75%) and reactions were accompanied by some hydrolysis of the ester substrate (3 – 8 %) (Fig. 25) [338]. Subtilisin A from *Bacillus licheniformis* (another alkaline serine protease), was used to convert peptide esters to peptide amides via ammonolysis in organic media with low water content and ammonium carbamate as an ammonia source. In this kinetically controlled conversion, the enzyme showed a high preference for non-polar amino acids at the *C*-terminus and the peptide sequence appeared to have a significant effect on the amidation/hydrolysis ratio (Fig. 26) [339]. Two more commercial Alcalases (Alcalase 2.4L FG and Alcalase 2.5L, type DX) were used as immobilized enzymes in sol-gel and tested in a batch and in a continuous reactor for the same conversion (Z-Ala-Phe-OMe to Z-Ala-Phe-NH_2, Fig. 26). Higher yields were obtained, but hydrolysis of the ester was still observed (10% in case of the continuous reactor system) [340].

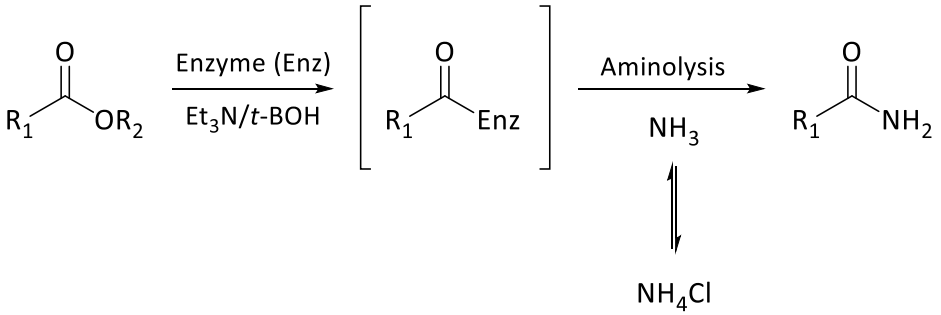


Fig. 25. *Mechanisms of amide formation catalyzed by Alcalase [338].*

Recently, subtilisin A was used as cross-linked enzyme aggregate (Alcalase-CLEA) to achieve better stability and activity in an anhydrous organic solvent. Peptides were added as ammonium salts. The enzyme preferred peptides over amino acids without hydrolyzing any internal peptide bonds with product yields up to 94%. The reaction media had low water content to avoid hydrolysis. Using this method, *N*-terminally protected, side-chain protected and fully unprotected peptides were amidated without any side reactions (Fig. 27) [341]. In the same study, lipase B from *Candida antarctica* (Cal-B) was also investigated to synthesize peptide amides after hydrolysis of peptides with Alcalase-CLEA initially observed. Cal-B catalyzed the amidation of *C*-terminal carboxylate groups of peptides in anhydrous solvent either with ammonium salts of α-carboxylic acids or using ammonia, the latter giving higher yields and faster reactions

(Fig. 28). However, Cal-B appeared limited in its substrate scope and bulky amino acids were not well accepted (e.g. Phe, Tyr, Trp, or Arg) [341].

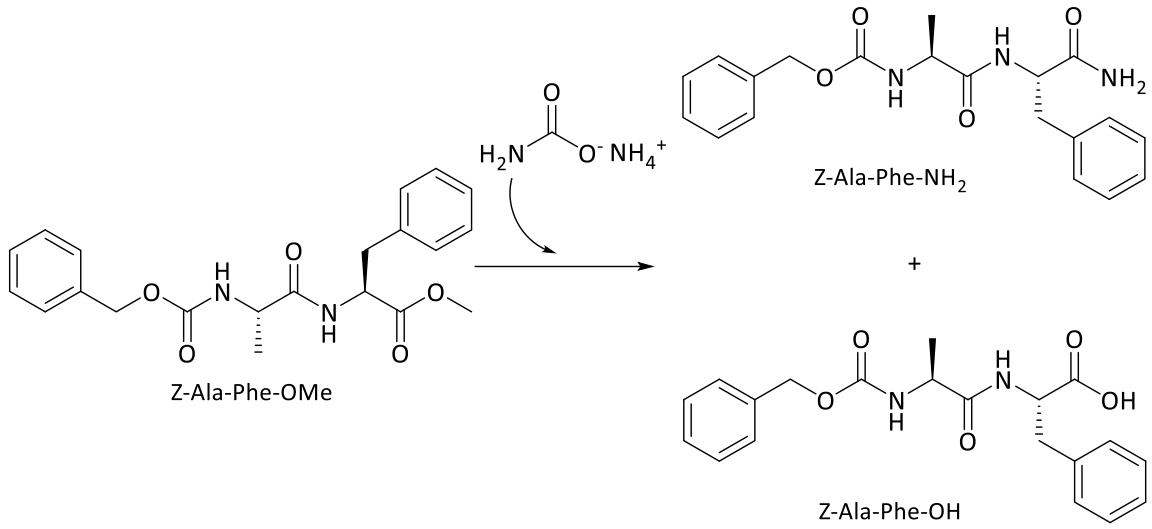


Fig. 26. *Kinetically controlled synthesis of Z-Ala-Phe-NH_2 by subtilisin A with 87% yield.*

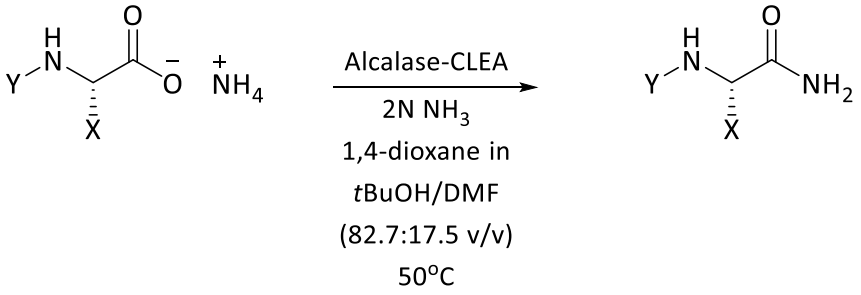


Fig. 27. *Alcalase-CLEA mediated amidation of peptide ammonium salts in organic media. Molecular sieves were added to the reaction mixture to maintain an anhydrous environment. X = amino acid side chain, Y = Peptide [341].*

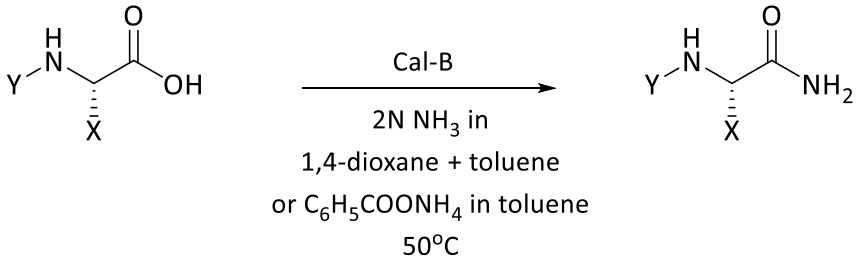


Fig. 28. *Lipase Cal-B mediated amidation of peptides in organic media. Molecular sieves were added to the reaction mixture to maintain an anhydrous environment. X = amino acid side chain, Y = Peptide [341].*

Another enzyme, peptide amidase from the flavedo of oranges, has also been investigated for *C*-terminal amidation of amino acids and peptides. In contrast to the proteases mentioned above for the amidation of the carboxy terminus, peptide amidase

could not be used for kinetically controlled amidation, owing to its lack of esterase activity. As the equilibrium favors hydrolysis in aqueous media, earlier attempts to use ammonium acetate in water-miscible solvents did not yield any amidation. However, in acetonitrile and small amounts (5%) of water, peptide amides were obtained by using the dipeptide Z-Gly-Phe-OH as the acyl component and ammonium carbonate or ammonium phosphate as the ammonium source (Fig. 29) [342]. Amidation occurred at a slightly alkaline pH which was controlled by the concentration of the nucleophile. The amidation reaction was limited in the substrate range (with a preference for bulky hydrophobic amino acids), and the conditions needed to be optimized for each substrate due to concurrent precipitation of peptides in the form of ammonium salts. Thus, the precipitated peptide substrates were excluded from the reaction mixture [342]. The synthesis was later optimized by adding as cosolvents DMF and DMSO to the acetonitrile-water mixture. Amidation of different *N*-protected di- and tripeptides was achieved but longer peptides were poorly accepted as substrates. Moreover, the poor solubility of some substrates and inactivation of the enzyme during the course of the reaction posed further challenges [343].

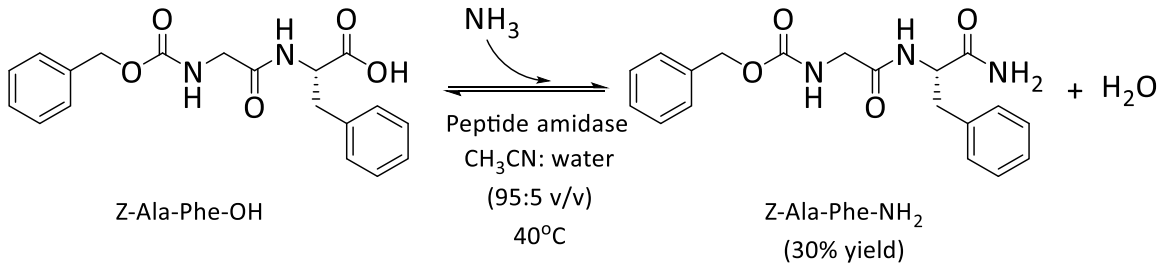


Fig. 29. *Peptide C-terminal amidation catalyzed by peptide amidase from flavedo of oranges. Y = peptide, X = amino acid side chain. The yield was later optimized by adding cosolvents to the system up to 60% [342,343].*

3.1.2.5 De-amidation

Besides hydrolysis of peptide esters, discussed above, carboxypeptidase Y can also hydrolyze peptide amides, similar to the native activity, with release of the *C*-terminal amino acid amide and expose a free carboxyl group on the *C*-terminus[344]. Deamidation of peptide amides can occasionally also be accompanied by ester formation, as found with Alcalase-CLEA. The reaction was performed in anhydrous medium with stabilizing cosolvents and fully selective amide to methyl ester interconversion was achieved in high yields (Fig. 30). Using this scheme, a chemotactic peptide antagonist, Boc-Met-Leu-Phe-OH, was synthesized in a stepwise manner where deamidation, esterification, and coupling steps were catalyzed by Alcalase-CLEA (Fig. 31). Apart from the broad substrate range of Alcalase, the applicability is deemed limited due to serious backbone alcoholysis in case of longer peptides [334]. In the same study, lipase B (Cal-B) was also found to

perform carboxamide to methyl ester interconversion of Cbz-Ala-NH_2, but interconversion of (di)peptides amides did not give promising yields [334].

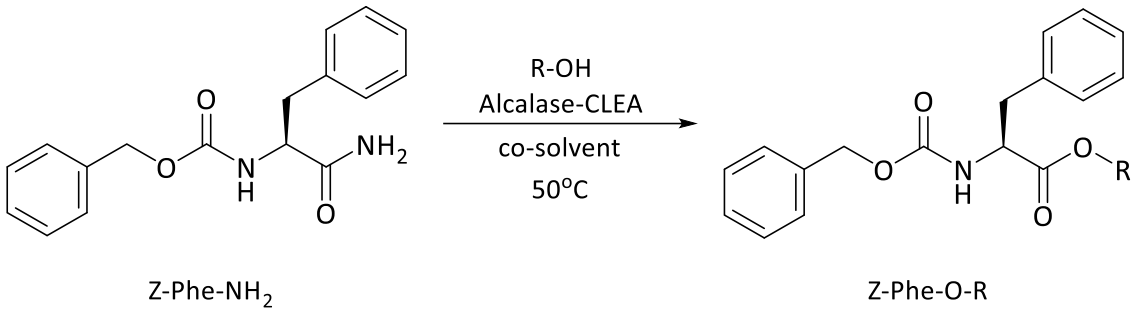


Fig. 30. *Alcalase-CLEA catalyzed conversion of Z-Phe-NH_2 into primary alkyl esters. R = methyl-, ethyl- or benzyl-. Reactions were performed either in MTBE, THF or DMF/THF (1/9, v/v), with added 3Å and 5Å molecular sieves. Yields: methyl ester, 98%; ethyl ester, 90%; benzyl ester, 83%.*

As mentioned earlier, protease-catalyzed modifications have inherent risks of peptide hydrolysis. Although proteases hydrolyze the carboxamide group more easily than a peptide bond, selective cleavage of the *C*-terminal protecting amide group is not straightforward because internal peptide bonds still are cleaved as well, making the discovery of better enzymes desirable. There are indications that more suitable enzymes exist. Peptide amidase isolated from the flavedo of oranges (peptide amidase) was shown to selectively hydrolyze peptide amides at the *C*-terminus with a broad substrate specificity (Fig. 32) [345,346]. The enzyme was also reported to catalyze the interconversion of di- tri- and tetra peptide carboxamides into the corresponding methyl esters, albeit with rather low yields of less than 40% [347]. Peptide amidase has also been applied for separation of racemic mixtures of *N*-terminally protected amino acid amides including non-proteinogenic amino acids. An example is the use of a microbial peptide amidase from *Stenotrophomonas maltophilia* that hydrolyzed *N*-acetyl-$_L$-neopentylglycine amide from a $_D/_L$-neopentylglycine amide mixture (Fig. 33) [348]. Such hydrolytic peptide amidases are discussed in more detail in the following sections because of their relevance to this thesis.

4.1 Peptide amidase

Steinke and Kula accidentally discovered the first peptide amidase enzyme from orange flavedo (*Citrus sinensis*) while purifying carboxypeptidase C in an attempt to obtain a novel enzyme for enzymatic peptide synthesis [349]. It was the first time that a selective enzyme was found that catalyzed the hydrolysis of the *C*-terminal amide group, releasing ammonia from peptide amides without hydrolyzing any internal peptide bonds. Earlier attempts to use amides as a *C*-terminal protecting group in peptides were problematic due to imminent partial hydrolysis catalyzed by proteases during amide hydrolysis. The enzyme accepted both *N*-protected amino acids and peptide amides as substrates, and was selective for L-amino acid amides. The enzyme was shown to retain

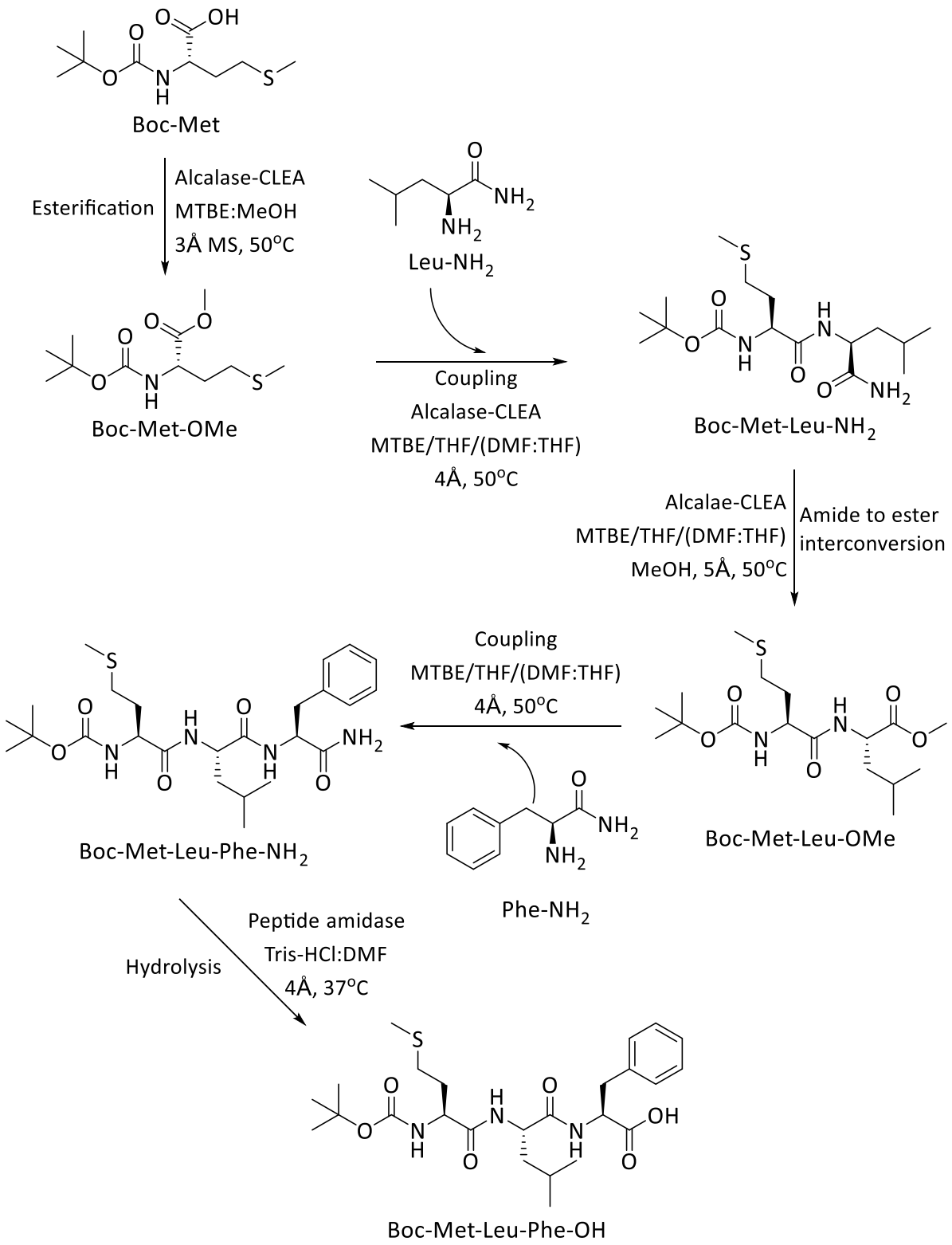


Fig. 31. *Alcalase-catalyzed steps during the synthesis of the chemotactic peptide antagonist Boc-Met-Leu-Phe-OH. All steps were catalyzed by Alcalase-CLEA while the last hydrolysis step was performed by peptide amidase. MS = molecular sieves.*

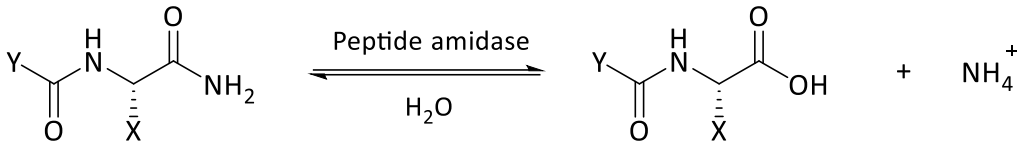
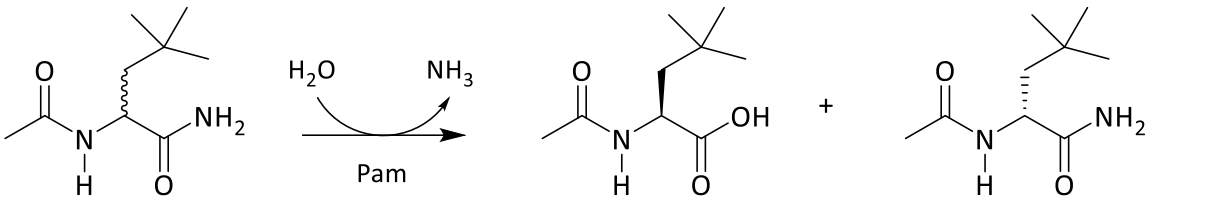


Fig. 32. *Hydrolysis of peptide amides by peptide amidase from orange flavedo. X = amino acid side chain, Y = amino acid- or peptide- residue, or N-terminal protecting group.*



N-Ac-D/L-neopentylglycine

N-Ac-L-neopentylglycine (99.8% enantiomeric purity)

N-Ac-D-neopentylglycine (99.7% enantiomeric purity)

Fig. 33. *Separation of racemic mixture of amino acid amides catalyzed by peptide amidase* [348].

around 30% of amidase activity in the presence of 30% dimethylformamide (used to solubilize peptides)[345]. Further investigations with partially purified peptide amidase showed that the enzyme had a wide substrate range, accepted a variety of di-, tri- and tetra-peptides, but not proline- and *N*-unprotected amino acid amides, tolerated D-amino acids at the penultimate position, and was not affected by *N*-terminal peptide modifications. Peptide amidase preparations had the highest level of activity when isolated from unripe oranges and fully selective amidase activities were recorded for a wide range of amino acid and peptide amide substrates. Even glycosylated peptide amides were deprotected. The rate of deamidation was reported to be low whenever the *C*-terminus was a bulky hydrophobic amino acid amide (e.g. Z-Trp-NH_2) [346].

In a proof of principle study, Schwarz *et al*. used the peptide amidase from orange flavedo (now denoted as PAF) in conjunction with carboxypeptidase-Y (CPD-Y) to synthesize Kyotorphin (a neuroactive peptide, H-Tyr-Arg-OH) in a continuous reactor where the two enzymes were separated by ultrafiltration membranes. CPD-Y catalyzed peptide bond formation between Tyr-OEt and Arg-NH_2. The resulting dipeptide amide (TyrArg-NH_2) was hydrolyzed to the free carboxyl terminus peptide (TyrArg) by PAF [350]. PAF was later studied further and characterized by Steinke *et al*. PAF was a 23±3 kDa protein, had an optimum pH of 7.5±1.5, optimum temperature of 30°C at pH 7.5, an isoelectric point of pH 9.5, and was stable from pH 6 to 9. PAF was also studied for the reverse reaction (i.e. amidation) as mentioned above [351]. The enzyme was believed to lack esterase activity, but Quaedflieg *et al*. later used PAF to convert oligopeptide amides to

oligopeptide esters [347]. PAF, however, is not well characterized to date and has not been obtained via microbial fermentation because the gene is unknown. Moreover, the yields obtained are low and the original method of extraction from orange peels makes it a laborious task to use it in peptide synthesis.

A microbial peptide amidase, from *Stenotrophomonas* (*Xanthomonas*) *maltophilia*, was discovered by screening strains known for deamidating carnitine amide against Z-Gly-Tyr-NH_2 as the substrate. This bacterial peptide amidase, Pam, had a native molecular mass of 38 ± 3 kDa and similar to PAF, did not contain any protease and peptidase activity. Also similar to PAF, Pam was stable at 30°C but had a different pH optimum (pH 5 to 6) and isoelectric point (5.8). Pam catalyzed deamidation of *N*-protected amino acid- or peptide amides. *N*-unprotected peptide amides were accepted as substrates but unprotected amino acid amides were not accepted. The enzyme showed a broad substrate range, but like with PAF, peptide amides with proline at the *C*-terminus were not converted. Pam was stereoselective for the *C*-terminus of peptides and amino acid amides, did not accept D-amino acid amides, which were however tolerated at the penultimate position. A high concentration of serine protease inhibitors was required to inhibit the enzyme suggesting a non-specific inhibition or weakly reactive serine. Similar to PAF, it was deemed that Pam would be a valuable tool for deamidation in a stepwise chemoenzymatic peptide synthesis scheme [352]. In a comparison study, Stelkes *et al*. used both PAF and Pam for the resolution of amino acid derivatives. *N*-acetyl aromatic amino acid amides (except *N*-acetyl-phenylglycine-NH_2) and also *N*-acetyl-Met-NH_2 were better substrates for Pam than the model substrate (Z-Gly-Tyr-NH_2). Apparently, a methylene group in the β-C position facilitated deamidation by rendering the side chain more flexible and lowering the steric constraints in the binding site of the enzyme. The enzyme gave the maximum yield of 50% with respect to the racemic substrate tested in the study [353].

Neumann *et al*. identified the Pam gene by Southern hybridization and cloned it in *E. coli* strain Origami DE3 [354]. Pam was found to be a periplasmic protein based on the *N*-terminal sequence. Sequence analysis also showed that Pam is a member of amidase signature family having a conserved amino acid signature sequence – GGSS[GS]G [355]. A truncated protein, devoid of the *N*-terminal signal sequence, was expressed and found to have better specific activity compared to the native enzyme isolated from *S. maltophilia*. The recombinant enzyme appeared to have a larger molecular weight (≈50 kDa in native state without the *N*-terminal sequence, in contrast to the originally reported MW of 38 kDa), and also had slightly different pH and temperature optima compared to previous data (pH 7.0–8.2 and 46–54 °C, in contrast to pH 5.0–6.5 and 37–45 °C). Substrate spectrum studies revealed that dipeptide amides were the best substrates while ultimate and penultimate amino acid residues had a major impact on the enzyme activity. Different versions of the recombinant enzyme based on *N*-terminal

or *C*-terminal truncations exhibited variations in activity [354]. Pam was expressed without the *N*-terminal sequence as a cytoplasmic protein with a *C*-terminal 6x His-Tag. The investigators further carried on to crystallize this peptide amidase and the X-ray crystallographic structure of Pam was obtained [356].

Both Pam and PAF were found to have a broad substrate range. From the literature, it appears that the substrate specificities for both enzymes overlap. Peptide amidases have a preference for dipeptide amides. With amino acid amides, PAF shows better activity than Pam while proline was not accepted by both amidases at all. The available data suggest that PAF prefers bulky groups at the penultimate position. Both enzymes prefer L-amino acids as amino acid amide substrate or within a peptide amide. Enzyme activity decreases in general by increasing the length of the amino acid amide chain [345,352–354].

4.1.1 Structure and mechanism of Pam amidase

Pam belongs to the amidase signature (AS) family of amidases, a highly diverse family of enzymes according to their substrate specificity and function (InterPro domain IPR023631). AS amidases catalyze amide bond ($CO\text{-}NH_2$) hydrolysis and are characterized by the presence of a highly conserved, serine/glycine-rich motif (GGSS[G/S]G) located in a highly-conserved stretch of ca. 130 amino acids, called the amidase signature sequence. The signature sequence region also holds the conserved Ser-(*cis*)Ser-Lys catalytic triad [357,358]. Examples of AS enzymes other than Pam are: 1) fatty acid amide hydrolase (hydrolyzes fatty acid amide substrates e.g. cannabinoid anandamide) [359], 2) malonamidase E2 (hydrolyzes malonamate) [358], and subunit A of Glu-tRNA(Gln) amidotransferase (catalyzes transamidation of misacylated Glu-tRNA(Gln) via amidolysis of glutamine) [360].

Pam has been crystallized with and without the inhibitor chymostatin (Cst). Its structure has been solved to 1.4 Å resolution [361]. Chymostatin inhibits serine-proteases by forming acyl-adducts with the serine nucleophile in the protein. In case of Pam, Chymostatin was found non-covalently bound in the active site. According to the crystal structure data, Pam folds in a conical structure consisting of double layers of α-helices surrounding a mixed 11-stranded β-sheet core. The AS domain is formed by a five-stranded β-sheet covered by four α-helices. The active site residues are present in a pocket located at the bottom of this fold (Fig. 34, and 35). The Ser-(*cis*)Ser-Lys catalytic triad is reminiscent of the classic Ser-His-Asp triad of serine hydrolases allowing the formation of an extended hydrogen-bond network between enzyme and substrate, and an oxyanion pocket that stabilizes the tetrahedral reaction intermediates. In AS enzymes, the unusual (*cis*)-Ser-Lys pair replaces the functional role of His-Asp, but the placement of the catalytic triad in the active site is different. It appears that the catalytic Lys can directly interact with the substrate which is important for substrate orientation in the active site, whereas in the classic Ser-His-Asp triad the Asp is buried deep in the protein and

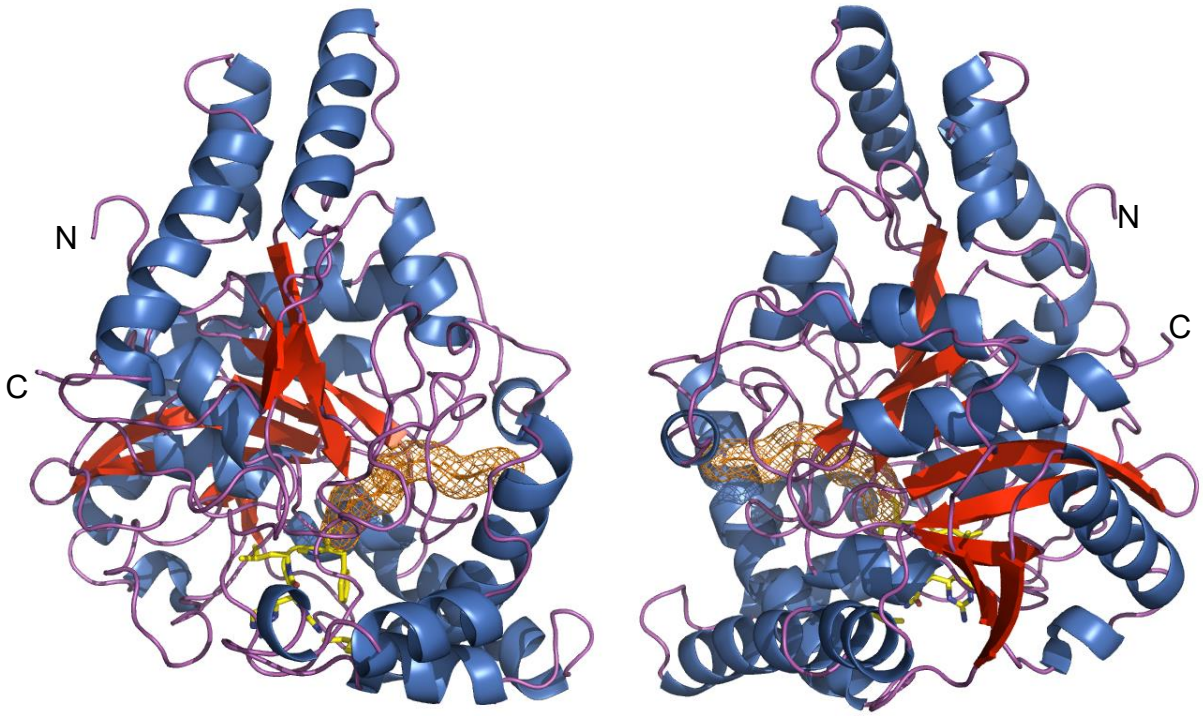


***Fig. 34**. Two views of the Pam 3D structure (rotated 180°) showing the secondary structure elements. The inner β-sheet core is colored in red, surrounded by α-helices in blue. The loops are colored purple. The inhibitor chymostatin is represented with a yellow backbone. A channel from the active site to the opposite site of the enzyme is represented as an orange mesh.*

interacts only with His [362]. Apart from similarities in structure, the AS amidases act upon a diverse range of substrates with different enzymes proposed to have a different mechanism for the formation of acyl-enzyme intermediate [361,363–365].

The description of the catalytic mechanism of Pam could be improved on the basis of computational studies on Pam and malonamidase E2 (MAE2) by Valina *et al*. and Cerqueira *et al*., respectively [362,363]. A general mechanism was proposed that details the proton transfer steps during the catalysis, and Fig. 36 outlines the most likely mechanism of peptide amidase (Pam). The essential feature is the formation of an acyl-enzyme intermediate that is subsequently hydrolyzed to yield the product. In the first chemical step, Ser226 performs a nucleophilic attack on the amide carbonyl carbon of the substrate. This reaction is facilitated by the hydrogen bond network between the catalytic Ser, (*cis*)Ser, and Lys residues. Ser226 becomes ionized by transferring its proton to (*cis*)Ser202 that in turn protonates Lys123. The oxyanion pocket, formed by backbone NH groups of Thr223, Asp224, and Ser226, stabilizes partial negative charge on the amide carbonyl oxygen, increasing its sensitivity to nucleophilic attack. As a result, the carbonyl carbon of the substrate becomes covalently bonded to Ser226 forming a negatively charged tetrahedral intermediate. The oxyanion pocket stabilizes the negative charge on the substrate carbonyl oxygen throughout the next step. The original proton of

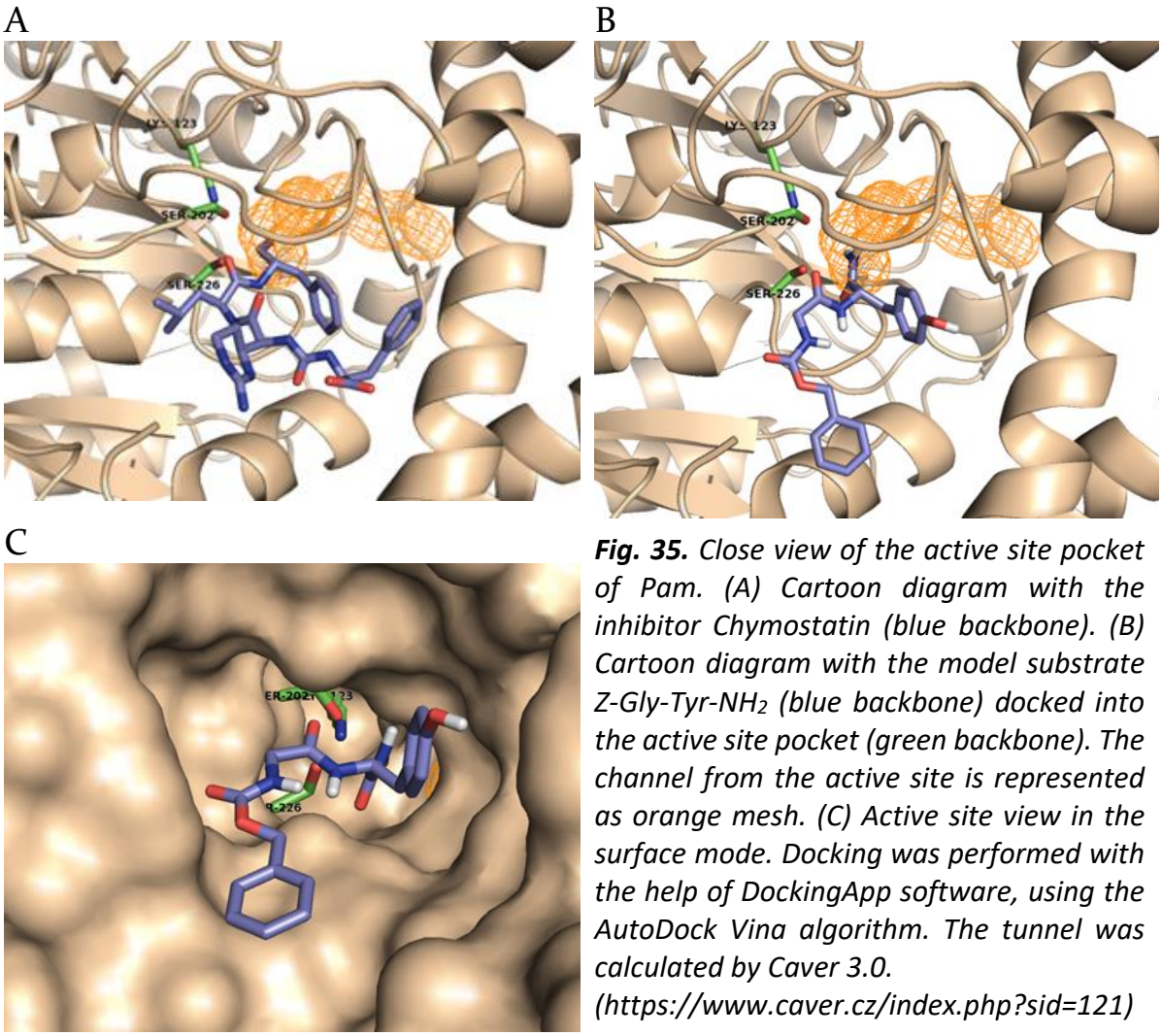


Fig. 35. *Close view of the active site pocket of Pam. (A) Cartoon diagram with the inhibitor Chymostatin (blue backbone). (B) Cartoon diagram with the model substrate Z-Gly-Tyr-NH_2 (blue backbone) docked into the active site pocket (green backbone). The channel from the active site is represented as orange mesh. (C) Active site view in the surface mode. Docking was performed with the help of DockingApp software, using the AutoDock Vina algorithm. The tunnel was calculated by Caver 3.0. (https://www.caver.cz/index.php?sid=121)*

Ser226 is now bonded with (cis)Ser202, which in turn donates its proton to Lys123, making the latter positively charged. In the second step, Lys123 transfers a proton to (*cis*)Ser202, which in turn protonates the amide (leaving) group of the tetrahedral complex while the negative charge on the carbonyl carbon is being stabilized by the oxyanion pocket. In the third step, the tetrahedral intermediate collapses, releasing NH_3 and forming the covalent acyl-enzyme intermediate. The hydrogen bonding network between the carbonyl oxygen of the substrate and the oxyanion pocket becomes weak.

The next two steps conclude the hydrolysis of the acyl-enzyme-intermediate, which requires a water molecule. The oxyanion hole stabilizes the covalent intermediate until hydrolysis. (cis)Ser202 protonates Lys123 and in turn is protonated by the water molecule. Simultaneously, the carbonyl carbon receives the hydroxyl group, the oxygen develops negative charge, which is now stabilized by Ser226, Asp224, and Thr223 of the oxyanion hole. In the last step, Lys123 protonates (cis)Ser202 which in turn protonates Ser226, detaching it from the acyl-enzyme intermediate. The resulting product, the peptide with a free carboxylic acid group, is released from the active site.

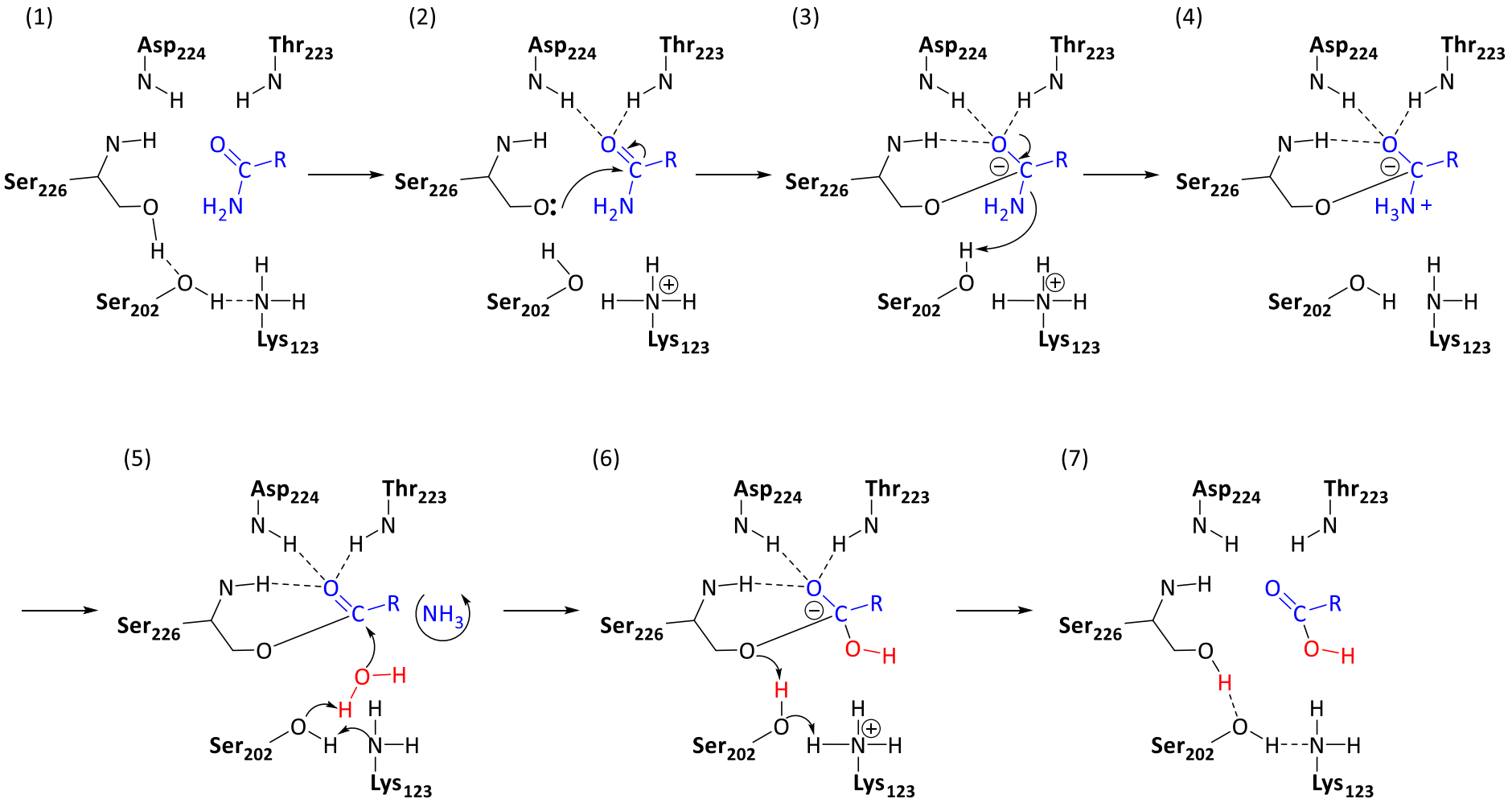


Fig. 36. *Proposed catalytic mechanism of Pam amidase. See text for details.*

5.1 Goal and outline of the thesis

The present work aims to discover more suitable enzymes for the modification of the *C*-terminus of peptides in order to facilitate the use of chemically synthesized peptides in enzymatic fragment coupling. Important reactions would be thermodynamically controlled amidation of the free carboxylate as well as kinetically controlled deamidation and esterification of the carboxamide moiety of peptide amides. A suitable biocatalyst should be resistant to organic cosolvents and active at low water content. Such an enzyme may catalyze deprotection and activation of a peptide via deamidation and esterification in a single step. Of the two peptide amidases that have been described in the literature, one (peptide amidase from the flavedo of oranges, PAF) has been reported to catalyze the desired methyl ester synthesis reaction, but hydrolysis was still the main conversion and large amounts of enzyme were required [347], indicating that better kinetic properties or improved activity at low water content is needed. Furthermore, neither this enzyme not any other plant peptide amidase has been biochemically characterized. The second known peptide amidase (Pam from *Stenotrophomonas*) has been cloned and expressed in bacteria but peptide *C*-terminal esterification has not been documented. An enzyme catalyzing the desired *C*-terminal modifications would be an ideal catalyst to materialize the *C*-terminal extension strategy for chemo-enzymatic peptide synthesis described above. Such an enzyme would also be useful for other reactions leading to C-terminal peptide modification, especially if they can be applied in neat organic solvents or solvent mixtures that facilitate peptide and reactant coupling.

Chapter 1 provides a general introduction to the field of peptides, their applications in health and food, and recent advances in the field of peptides synthesis. Strategies for peptide synthesis are reviewed and different ways to achieve protection and deprotection of amino acids and peptides are also described. In the last section of this Chapter, we discuss peptide amidases available to date and in particular, the peptide amidase from *Stenotrophomonas maltophilia* is discussed, which is the only peptide amidase of which a crystal structure has been reported.

Chapter 2 describes the discovery and characterization of a peptide amidase from *Glycine max* (soybean) by cloning cDNA. It was identified as a homolog of a peptide amidase that we detected by mass spectrometry in partially purified protein preparations of *Citrus* flavedo or in a commercial preparation of peptide amidase.

Chapter 3 presents the use of peptide amidase Pam from *Stenotrophomonas maltophilia* for esterification of peptides and shows the use of enzymatically esterified peptide in peptide coupling mediated by a subtilisin variant.

Chapter 4 discusses the development of a highly thermostable Pam using computational design and screening of stabilizing mutations. Beneficial mutations are

discovered and combined, leading to a highly stable and solvent compatible enzyme. Different coupling reactions that can be performed by the stabilized enzyme and its use in organic solvents are discussed as well.

Chapter 5 discusses mutagenic studies to improve the thermostability of the peptide amidase from *Glycine max*. The results show that stability can be enhanced by the introduction of mutations that replace amino acids that are very different from consensus in the native sequence.

The thesis concludes with a general discussion in **Chapter 6**. A review of the findings reported in this thesis and the role of peptide amidases in chemo-enzymatic peptide synthesis will be discussed.

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